DETERMINATION OF NATURAL STEROIDAL ESTROGENS IN FLUSHED DAIRY MANURE WASTEWATER AND SURFACE AND GROUNDWATER

By

TRAVIS A. HANSELMAN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2004

ACKNOWLEDGMENTS

The author thanks Dr. D.A. Graetz, chair of the supervisory committee, for providing the opportunity, research facilities, and equipment to conduct the research and for his patience and guidance throughout the project. The author thanks cochair Dr. A.C. Wilkie for thorough editing of the dissertation work, research facilities and equipment, and supportive counseling during this academic pursuit. Sincere appreciation is extended to Drs. T.A. Obreza and N.D. Denslow for their comments and suggestions and for participation on the supervisory committee. The author thanks Dr. N. Szabo and the staff of the Analytical Toxicology Core Laboratory at the University of Florida for their hard work involving mass spectrometry analysis. The author is grateful for the research funding provided for the project by the UF School of Natural Resources and Environment Mini-Grants program.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DETERMINATION OF NATURAL STEROIDAL ESTROGENS IN FLUSHED DAIRY MANURE WASTEWATER AND SURFACE AND GROUNDWATER

By

Travis A. Hanselman

May 2004

Chair: Donald A. Graetz
Cochair: Ann C. Wilkie
Major Department: Soil and Water Science

Estrogens are an environmental concern because low ng L^{-1} concentrations in water can adversely affect aquatic vertebrate species by disrupting the normal function of their endocrine systems. There is a critical need to accurately measure the concentrations of estrogens in dairy wastes—a potential source of estrogens to waterways. At present, however, there is a lack of suitable analytical techniques for measuring estrogens in dairy wastes and waste-impacted water resources. Therefore, the objective of this research was to develop methods to measure estrogens including estrone, 17α-estradiol, 17ß-estradiol, and estriol in flushed dairy manure wastewater (FDMW) and in surface and groundwater.

Enzyme immunoassay and gas chromatography-mass spectrometry (GC-MS) analytical methods for the measurement of estrogens were studied. Analysis of 17ß-estradiol by three immunoassays revealed that matrix effects significantly affected the accuracy of one or all of the immunoassays. An extensive sample preparation method involving chromatographic purification was deemed necessary so that estrogens could be

measured by GC-MS. A new method was developed that enabled low ng L^{-1} measurements of estrogens in FDMW. Three estrogens were measured in FDMW: estrone, 17α-estradiol, and 17ß-estradiol. Estriol was not detected in FDMW.

To address concerns regarding possible contamination of surface and groundwater at a dairy, the new method was adapted for water samples and a survey experiment was conducted. During method development, it was found that interference affected the GC-MS quantification of estrogens in water samples. However, the sample preparation method appeared promising because, after accounting for interference, excellent extraction recoveries were observed. Measurable concentrations of 17α-estradiol, 17ß-estradiol, or estriol were not found in surface or groundwater at the dairy. Some estrone was detected in surface water that was directly impacted by cattle. However, a similar concentration of estrone was also measured in groundwater from a non-impacted location. Further refinement and validation of the method is needed for more conclusive studies of estrogens in manure-impacted water.

CHAPTER 1
INTRODUCTION

Livestock manure contains appreciable amounts of natural steroidal estrogen hormones, particularly estradiol, estrone, and estriol, that can potentially contaminate surface and groundwater resources (*1-8*). Estrogen contamination of water is a concern because low part per trillion (10 to 100 ng L^{-1}) concentrations of these chemicals can adversely affect the reproductive biology of aquatic wildlife such as fish, frogs, and turtles by disrupting the normal function of their endocrine systems (*9,10*). For example, concentrations of 17ß-estradiol or estrone in water $\geq$30 ng L^{-1} for 21 days induced vitellogenin (an egg yolk precursor protein that is normally produced only by adult females) synthesis and abnormal testicular growth in male fathead minnows (*Pimephales promelas*) (*11,12*).

Few researchers have measured the impact of manure-borne estrogens on fish and wildlife, but Irwin et al. (*13*) studied the concentrations of 17ß-estradiol in farm ponds impacted by beef cattle runoff and the effect of estradiol on vitellogenin production in painted turtles (*Chrysemys picta*). 17ß-Estradiol concentrations in the ponds ranged from <1 to 7 ng L^{-1}. Juvenile and male turtles did not synthesize vitellogenin during 28 d of exposure, but female turtles collected from the runoff-impacted ponds had significantly greater concentrations of vitellogenin than female turtles from nonimpacted (control) ponds.

Clearly, it is important to have accurate information about the occurrence of estrogens in dairy wastes so that any estrogen contamination of surface and groundwater

resources can be prevented or minimized. At present, however, there is a lack of suitable analytical techniques for studying the occurrence and fate of estrogens in livestock wastes and impacted waterways. Therefore, the objective of this research was to develop methods for the measurement of estrone, 17α-estradiol, 17ß-estradiol, and estriol in flushed dairy manure wastewater (FDMW) and surface and groundwater.

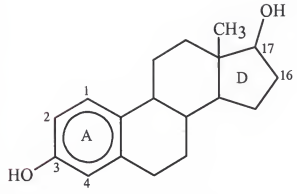
The subsequent chapters presented in this dissertation were prepared as individual manuscripts. In this chapter, the research problem and objective were identified. Chapter 2 is a literature review of the physicochemical properties of steroidal estrogens, analytical methods, livestock excretion, and the fate of manure-borne estrogens in the environment. In chapter 3, some limitations of enzyme immunoassay for measuring 17ß-estradiol in FDMW are described. Chapter 4 details a new sample preparation method that enabled the measurement of estrogens in FDMW by GC-MS. The new method was modified in chapter 5 and used for a preliminary survey of estrogens in surface and groundwater at a dairy farm. Chapter 6 provides a summary and conclusions of the results presented in the previous chapters.

CHAPTER 2
LITERATURE REVIEW

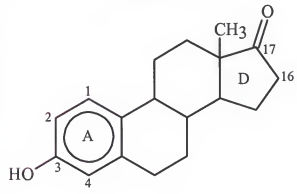
The objective of this chapter is to assess the current state of science regarding estrogen physicochemical properties, analytical methods, livestock excretion, and the biogeochemical fate of manure-borne estrogens in the environment for the purpose of identifying priority research needs. The scope of this review is limited to the natural estrogen steroids estradiol, estrone, estriol, and their conjugated metabolites. The trivial names and systematic nomenclature for the main chemical compounds that are described in this text are as follows: 17α-estradiol (1, 3, 5(10)-estratrien-3, 17α-diol), 17ß-estradiol (1, 3, 5(10)-estratrien-3, 17ß-diol), estrone (1, 3, 5(10)- estratrien-3-ol-17-one), estriol (1, 3, 5(10)- estratrien-3, 16α, 17β-triol).

Structure and Physicochemical Properties

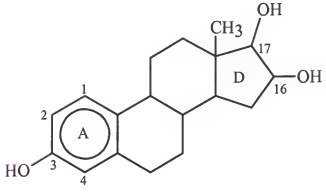
Estradiol, estrone, estriol, and other natural steroidal estrogens contain an aromatic A-ring as a distinctive part of their tetracyclic molecular framework (Figure 1) (*14,15*). Key structural differences arise in the D-ring structure owing to the type and stereochemical arrangement of functional groups at the C-16 and C-17 positions. Estradiol can have either a hydroxyl group at C-17 that points downward from the molecule (α configuration) or a hydroxyl group that projects upward from the molecule (ß configuration). Estrone differs from estradiol because there is a carbonyl group at C-17 rather than a hydroxyl. Estriol features hydroxyl groups at both the C-16 and C-17 position and, thus, has four epimers. Conjugated estrogens are analogous in structure to



Estradiol



Estrone



Estriol

Figure 2-1. Molecular structures of estradiol, estrone, and estriol. The letters and numbers indicate the ring assignments and carbon numbers, respectively.

estradiol, estrone, or estriol, except that a sulfate and/or glucuronide group is substituted at the C-3 and/or C-17 positions of the parent compound (e.g., 17ß-estradiol-3-sulfate, 17ß-estradiol-17-sulfate, 17ß-estradiol-3,17-disulfate). An in-depth description of the electronic structure, crystal geometry, and spectral characteristics of the different estrogens is beyond the scope of this review but is available in Salole (*16*) and Kubli-Garfias (*17*).

The physicochemical properties of estradiol, estrone, and estriol are given in Table 1. Tabak et al. (*18*) reported that the solubility of 17ß-estradiol, estrone, and estriol, in water was 13.3 mg L^{-1}, 12.4 mg L^{-1}, and 13.3 mg L^{-1}, respectively. The temperature associated with the solubility data was not provided. Considerably lower aqueous solubility estimates were reported by Hurwitz and Liu (*19*). They determined that the solubility of 17α-estradiol, estrone , and estriol at 25° C was 3.9 mg L^{-1}, 0.8 mg L^{-1}, and 3.2 mg L^{-1}, respectively. A mid-range value was reported by Batra (*20*), who reported that the solubility of 17ß-estradiol at 23-24° C was 7.0 mg L^{-1}. Estradiol solubility doubled, however, when progesterone was added into the solution. This result suggests a mutual effect of other substances on the solubility of estradiol. Similar results were found by Hahnel (*21*), who reported enhanced solubility of 17ß-estradiol in phosphate buffer in the presence of some amino acids such as arginine, aspartic acid, glutamic acid, lysine, tryptophan, tyrosine, proline, and histidine. The aqueous solubility of estrogens can also be greatly enhanced by surfactants like Tween 20, polysorbate 40, tetradecyltrimethylammonium bromide, and sodium dodecyl sulfate (*22-24*). For instance, Blomquist and Sjoblom (*23*) solubilized ~150 mg L^{-1} and ~300 mg L^{-1} of estradiol and estrone, respectively, in a 0.08 M aqueous solution of Tween 20 (at 20°C).

The solubility of unconjugated estrogens has also been measured in various organic solvents. Estradiol and estrone are more soluble in polar solvents such as acetone than nonpolar solvents such as hexane (*25-27*). Ruchelman and Haines (*26*) reported that 17ß-estradiol and estrone solubilty in acetone (at 30°C) was about 89 g L^{-1} and 17 g L^{-1}, respectively. Information about the distribution of estrogens between immiscible solvents such as ether and water is provided by Mather (*28*).

Literature values for the log octanol-water coefficients (log K_{ow}) of estrogens range from 3.1 to 4.0 for 17ß-estradiol, 3.1 to 3.4 for estrone, and 2.6 to 2.8 for estriol (*29-31*). The coefficients suggest that estradiol and estrone are about equally hydrophobic and that estriol is the least hydrophobic of this group. In a more general way, these numbers indicate that the steroidal estrogens are moderately hydrophobic compounds. The practical usefulness of log K_{ow} as it relates to the prediction or modeling of the partitioning of estrogens between solid and liquid phases in the environment has not been extensively studied. However, Furhacker et al. (*32*) concluded that octanol-water partition coefficients were not useful to predict the behavior of 17ß-estradiol at environmentally relevant concentrations since 95% of added 17ß-estradiol (spiked to 50 ng L^{-1}) remained in an aqueous phase after a 24h equilibration period with 128 mg L^{-1} suspended solids from a wastewater treatment plant. Conversely, however, Lai et al. (*29*) found the coefficients useful for predicting estrogen sorption to sediments in river and estuarine systems.

Hurwitz and Liu (*19*) determined the ionization constants (pK_a values) of 17α-estradiol, estrone, and estriol to be ~10.5, 10.3, and 10.4, respectively. Slightly greater pKa values for 17ß-estradiol (10.7) and estrone (10.8) were reported by Lewis and

Table 2-1. Selected physicochemical properties of steroidal estrogens.

Property	Estradiol	Estrone	Estriol	Reference
Formula	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_3$	
MW (g mol^{-1})	272.4	270.4	288.4	(*29*)
S_w (mg L^{-1})	3.9—13.3	0.8—12.4	3.2—13.3	(*18-20*)
VP (Pa)	3×10^{-8}	3×10^{-8}	9×10^{-13}	(*29,30*)
log K_{ow}	3.1—4.0	3.1—3.4	2.6—2.8	(*29-31*)
pK_a	10.5—10.7	10.3—10.8	10.4	(*19,33*)

MW, molecular weight; S_w, solubility in water; VP, vapor pressure; K_{ow}, octanol-water partition coefficient; K_a, acid ionization constant.

Archer (*33*). These values indicate that estrogens are weak acids and that ionized species would not be expected under normal environmental pH conditions.

The vapor pressures of the natural estrogens are in the range of 9×10^{-13} to 3×10^{-8} Pa (*29,30*). These numbers indicate that the volatilization of estrogens is negligible and that gaseous measurements of estrogens are not needed for experimental mass balance. Therefore, studies of the environmental fate of estrogen steroids can be limited to their behavior in terrestrial and aquatic systems.

Physicochemical data for conjugated estrogens were not found in the literature. However, estrogen conjugates likely have much greater aqueous solubility than unconjugated estrogens due to their polar glucuronide or sulfate functional groups.

Analytical Overview

The accurate determination of steroidal estrogen hormones in complex matrices like manure, wastewater, soil, and water is a difficult and expensive task that requires the skillful application of highly sensitive and selective analytical procedures. Some reviews are available regarding chemical analysis of estrogens in biological and environmental matrices (*34*). The following information is a summary of the major sample preparation

steps that are normally involved for the analysis of estrogens and provides some information about the sensitivity of the major quantification techniques.

Sample Preservation and Handling

Sample preservation is critical to avoid losses of estrogens via chemical or microbial transformations (*35-40*). Several authors have used cold storage, i.e., refrigeration at 4° C or freezing at -20° C for preservation (*3,41-46*). Raman et al. (*6*) reported that, in addition to cold storage (5° C), acidification with H_2SO_4 to pH ~2 was also needed to preserve estrogens in dairy waste samples. Alternatively, Terio et al. (*45*) found that fecal samples could be stored in 95% ethanol for up to 14 d at room temperature without significant estrogen losses. Baronti et al. (*47*) compared the stability of estrogens in bottled river samples without the addition of a preservative agent, the storage of samples with formaldehyde (1%), and the storage of estrogens on Carbograph solid phase extraction (SPE) sorbent. They found severe losses of estrogens during 7 days of storage at 4° C when no preservatives were added to river water samples. Formaldehyde prolonged estrogen stability for up to 28 d, but the best strategy for avoiding estrogen degradation was passing the river water samples through the Carbograph sorbent, then washing the cartridge with methanol to eliminate bacterial contamination and storing the device at –18° C. Using this procedure, they demonstrated that 89% of 17ß-estradiol, 93% of estrone, and 92% of the estriol that was added into the samples could be recovered from the cartridges after 60 d of storage.

Care must also be taken to avoid losses of estrogens due to sorption onto laboratory equipment, "creepage" phenomena, and decomposition by exposure to air. Jurgens et al. (*48*) measured the sorption of 17ß-estradiol to glass, polytetrafluoroethylene (PTFE), polycarbonate, and polypropylene containers. Glass and

PTFE containers sorbed less than 1% of 17ß-estradiol, from solution (concentration not specified) after 2 days of equilibration. The greatest sorption of 17ß-estradiol (4%) occurred on polypropylene tubes. Batra (*20*) found that glassfibre filters sorbed much less 17ß-estradiol (3%) than membrane filters (24%). Kushinsky and Anderson (*49*) reported significant losses of estrogens from samples that were stored in glass vials as a result of creepage along vessel walls and subsequent chemical decomposition by air into more polar compounds. Glassware silanization was effective in reducing the creepage problem and was later recommended by Cohen et al. (*50*), Fotsis and Adlercreutz (*51*), and Jarvenpaa et al. (*52*) for preparing urine samples for estrogen analysis. Significant losses of estrogens as a result of exposure to air was also found by Coyotupa et al. (*53*), Doerr (*54*) and Kushinsky (*55*) during thin-layer chromatography separations with silica gel.

Hydrolysis of Conjugates

Several methods (enzyme hydrolysis, acid solvolysis, methanolysis, and ammonolysis) are reported in the literature for hydrolyzing estrogen conjugates, but complete deconjugation is rare (*18,56-59*). A few researchers have compared the effectiveness of different hydrolysis methods (*56,59-61*). For example, Bain et al. (*56*) showed that ammonolysis (anhydrous liquid ammonia, -35° C, 1 M HCl pH 2) gave very efficient recoveries of estradiol-3,17-disulfate compared with acid solvolysis (2×10^{-6} M sulfurinc acid in ethyl acetate, 30° C, 18 h) or enzyme hydrolysis (ß-glucuronidase/arylsulfatase 37° C, 24 h), with a net hydrolysis of 89, 11, and 1%, respectively. Generally, enzyme hydrolysis is preferred to acid hydrolyis due to the possibility of steroid degradation via dehydration of the hydroxl group at the C-17 position (*62,63*). However, enzyme hydrolyis can be inhibited by substances in urine (*51,57*). Furthermore, endogenous bacteria in non-sterile samples like manure may

reduce the effectiveness of the added enzymes or result in degradation of the liberated unconjugated estrogens during the long incubations (e.g., 24 h) required for hydrolysis.

Tang and Crone (*59*) reported a methanolysis deconjugation method that avoids some of the problems associated with acid or enzyme hydrolysis. Finlay-Moore et al. (*3*) attempted the methanolysis procedure to measure conjugates in poultry manure-impacted runoff water. With pure solutions, estradiol-3-sulfate and estradiol-17-ß-glucuronide were cleaved, but estradiol-3,17-disulfate was not. Methanolysis proved unsuccessful for runoff samples, however, since measured values increased ≤150% in some cases and decreased ≤63% in other samples.

Extraction

The extraction of unconjugated estrogens from solid samples like soils, sediments, and lyophilized manure has been accomplished with a variety of solvents including ethanol, methanol, acetone, ethyl acetate, ether, chloroform, and toluene (*3,45,64-67*). Sequential extractions with methanol, acetone, or ethyl acetate gave high extraction efficiencies (70 to 103%) for both soils and sediments (*64,67*).

Some researchers have reported the use of deionized water, phosphate buffer, or aqueous solutions of NaCl to accomplish the extraction step, but reported no extraction recovery percentages of spikes to the matrix (*1,5,41,68*). Thus, it is not known if aqueous solvents are effective extractants for estrogens. Based on the low aqueous solubility and moderate hydrophobicity of estrogens, it seems doubtful that water or salt solutions would be effective extractants.

Liquid-liquid extraction (LLE) is a traditional approach for the extraction of estrogens and other steroids from aqueous suspensions and fluids. Raman et al. (*6*) used LLE with ether for the extraction of estrogens from dairy waste. Details regarding the

recovery of fortified samples were not reported, but using a similar approach involving LLE with ether, Vos et al. (*46*) reported recovery percentages of 86, 85, and 72% for 17ß-estradiol, estrone, and estriol, respectively, from swine fecal suspensions. Lai et al. (*29*) used dichloromethane for LLE of estrogens from surface water. Recoveries of added 17ß-estradiol, estrone, and estriol (0.1 $\mu g\ mL^{-1}$) were about 82, 83, and 81%, respectively. Tabak and Bunch (*69*) used chloroform for LLE of estrogens from culture media and reported a recovery percentage of 97% to 100%.

During the last several years, solid-phase extraction (SPE) has become more widely used than LLE for separating estrogens from aqueous samples. The most popular sorbents used in both column and disk SPE formats contain octadecylsilica (C18), polymerics like styrene divinylbenzene (SDB), graphitized carbon black (GCB), or some combination of functionalities (*47,67,70-80*). Most studies using SPE for estrogen extraction from wastewater have reported better than 80% recovery of estrogens (*81*). Theoretical and practical information regarding the optimum sample processing conditions for the solid-phase extraction of estrogens can be found in Hennion (*82*), Lopéz de Alda and Barceló (*67*), and Seibert and Poole (*77*). In addition to extraction, SPE is also used for sample purification (more details below).

Sample Purification

Ideally, the primary extraction step— accomplished by liquid or SPE—yields a sample that is sufficiently pure for analysis. In reality, however, the extracts of manure, soil, and natural water contain an abundant and diverse array of organic and inorganic substances that can interfere with estrogen quantification (*6,67,73*). Therefore, an advanced sample purification (clean-up) technique should be considered mandatory. The degree of sample purification that is needed will depend on the complexity of the sample

matrix involved, the analytical accuracy and sensitivity desired, and practical considerations like the amount of time, money, and effort required to validate the purification technique.

Solid-phase extraction (SPE) is an effective and practical purification technique and a has generally replaced traditional separation techniques like solvent partitioning, paper chromatography, and thin-layer chromatography for purification of complex biological samples (*18,82-86*). Some researchers use SPE in combination with high-performance liquid chromatography (HPLC) for a very rigorous sample purification prior to analysis. For example, Snyder et al. (*78*) used SDB SPE for extracting 17ß-estradiol from wastewater effluent and surface water. The SDB extract was purified using normal-phase HPLC for fractionation prior to analyis by radioimmunoassay (RIA). Similarly, Huang and Sedlak (*73*) extracted 17ß-estradiol from municipal wastewater effluent and surface water by SPE with C18. The C18 eluant was further fractionated by HPLC to remove organic matter from the samples prior to estrogen analysis.

Quantification

Colorimetric and fluorometric methods were once used extensively for the measurement of estrogens in urine and feces (*87-97*). Unfortunately, many interferences were often noted with the color reactions, and tedious sample preparations were necessary to achieve reliable data (*98*). Chromatographic purification of the samples resolved some issues regarding sensitivity, but the extensive manipulation of the samples often resulted in high losses of the analytes (*95,99,100*). For example, Mathur and Common (*95*) reported that the smallest amount of 17ß-estradiol that could be measured was 0.7 $\mu g\ 24\ h^{-1}$ for duplicate determinations of urine extracts from chickens after

separation by TLC on Silica Gel G (Merck) and measurement by colorimetry. However, the average percentage recovery of added 17ß-estradiol was only about 35%.

Gas chromatography (GC) techniques gradually replaced colorimetric methods for the analysis of estrogens during the mid 1960's. Jones and Erb (*101*) used gas-liquid chromatography (GLC) coupled with a flame ionization (FI) detector system for the analysis of estrogens in livestock urine. The minimum amounts of estradiol and estrone that could be quantified with their system were 0.01 µg and 0.05 µg, respectively. Tang et al. (*102*) used GC-FI to characterize the urinary estrogen metabolites of the domestic chicken; the smallest amount of an estrogen that could be detected was 0.3 µg. Tabak et al. (*18*) used GLC to provide some of the first information about the persistence of estrogens in municipal treatment plants, but did not clearly state the detection limits associated with their procedure.

Today, a number of GC and LC mass spectrometry (MS) and tandem mass spectrometry (MS-MS) methods have been proposed for the analysis of estrogens in sewage, sewage effluent, and water samples (*47,67,78-80,103-107*). These techniques may be useful, if not directly applicable, for the quantification of estrogens in livestock wastes and waste-impacted soils and waterways. The sensitivity of the GC-MS or LC-MS analysis of environmental matrices depends on the equipment used, the origin of the sample tested, and the degree of sample purification for removing interferences from the matrix. For example, Spengler et al. (*79*) reported GC-MS detection limits ranging from 0.4 to 0.7 ng L^{-1} for estrogens in sewage effluent samples that were extracted using C18 SPE and then purified using silica gel. Raman et al. (*6*) reported GC-MS detection limits for ether extracts of dairy manure (no clean-up) of about 10 µg L^{-1}. Lower detection

limits have been reported using tandem mass spectrometry (MS-MS) and other sophisticated detectors. Fine et al. (*103*) developed a method for quantifying estrogens in groundwater and swine lagoon wastes. Estrogens were extracted and purified using a Supelco Oasis HLB cartridge. They reported a limit of quantitation of 1 and 40 ng L^{-1} in groundwater and swine wastes, respectively. Huang and Sedlak (*73*) reported GC-MS-MS detection limits in the range of 0.2 to 0.4 ng L^{-1} for the analysis of HPLC-purified wastewater effluent samples. Ternes et al. (*80*) achieved GC-MS-MS detection limits of 0.5 ng L^{-1} for surface water, and 1 ng L^{-1} for raw and treated sewage, samples purified using silica gel. Kuch and Ballschmiter (*75*) determined estrogens in surface and drinking water by HRGC-NCI-MS (high resolution gas chromatography with negative chemical ionization mass spectrometric detection in the selective ion mode). They reported detection limits of 0.05 ng L^{-1} and 0.2 ng L^{-1} for estrogens in drinking water and sewage effluent, respectively. Similar work by Nakamura et al. (*108*) using GC-NCI-MS for the analysis of river water samples reported detection limits of 0.1 to 0.3 ng L^{-1}.

Liquid chromatography systems equipped with MS, MS-MS, or other sophisticated detectors are also used for estrogen analysis (*81,109*). However, a significant limitation of the LC-MS or LC-MS-MS systems for analyzing manure samples is the potential for ion suppression due to sample matrix effects (*103*). Nevertheless, excellent detection limits have been reported in a variety of environmental samples. Ferguson et al. (*110*) reported detection limits of 0.1 ng L^{-1} for estrone and 0.2 ng L^{-1} for 17ß-estradiol, using HPLC with electrospray MS detection for the analysis of sewage effluent. Baronti et al. (*47*) used LC-ESI-MS-MS (LC coupled with negative turbo ion spray tandem mass spectrometry in selected reaction monitoring mode) to monitor estrogens in sewage

treatment plants and river water. The limits of quantification ranged from 0.01 ng L^{-1} for estrone in river water to 0.6 ng L^{-1} for both estradiol and estriol in sewage influent. Lopez de Alda and Barcelo (*104*) reported detection limits of 10, 10, and 15 ng L^{-1} for estriol, estradiol, and estrone, respectively, using LC with a diode array detection system (DAD) for the analysis of drinking water. However, for samples obtained from highly polluted surface water and sewage effluent, accurate quantification was possible only at concentrations ≥200 ng L^{-1} due to the inherent complexity of the samples that were analyzed and the lack of an extensive purification protocol. Matsumoto et al. (*111*) derivatized estrogens using a ß-diketonate europium chelate and used HPLC with a time-resolved flourimetric detection system for the analysis of river water samples. The signal for estriol could not be resolved due to the matrix effects of the river water samples, but they reported a detection limit of 1.6 ng L^{-1} for both 17ß-estradiol and estrone.

Immunoassay methods of quantification are attractive alternatives to the aforementioned chromatographic techniques because equipment costs are relatively low, few specialized skills are needed by the analyst to perform the assay, and low detection limits can be achieved. However, the accuracy and reliability of the immunoassay system can be compromised by interferences due to cross reactivity, enzyme inhibition, matrix effects (pH, ionic strength), endogenous enzymes, and chromagens (*112-115*). Once the interfering compounds are removed from the samples, however, some immunoassay techniques can provide results that are comparable with those obtained by GC-MS-MS (*73*).

Estrogens can also be measured using in vitro or in vivo biological assays. However, bioassay quantitation methods are fundamentally different than the

abovementioned chemical methods of quantitation since they measure total estrogenic activity via a biological response. By convention, bioassay systems are calibrated with 17ß-estradiol (the most potent of the natural estrogens) and the measured response is reported as estradiol equivalent units, or some other relative term.

Popular in vitro methods for environmental analysis include yeast-based screening assays, recombinant receptor-reporter assays, cell proliferation assays, and receptor binding assays (*74,76,116-122*). In vitro bioassays are widely used for detecting the estrogenic activity of environmental samples, but some samples may contain substances such as humic acids, pesticides, and antibiotics that interfere with the analysis (*123*). For example, Raman et al. (*6*) found that concentrated extracts of dairy waste were toxic to the *Saccharomyces cerevisiae* strain of yeast used in the YES (yeast estrogen screen) assay. Burnison et al. (*124*) reported a method for identifying estrogenic substances in hog manure and manure-impacted tile drainage water with the YES bioassay and rainbow trout estrogen receptor assays. In addition to 17ß-estradiol and estrone, they found that equol (a phytoestrogen) was a significant source of estrogenicity in hog manure. The detection limits associated with in vitro bioassays vary. Murk et al. (*76*) compared an estrogen receptor binding assay (rat uterus cytosol containing an estrogen receptor) with YES assay and the ER-CALUX (estrogen receptor-mediated luciferase reporter gene) assay for measuring estrogenic potency of wastewater and surface water extracts. All three assays detected estrogenicity, but the detection limits for 17ß-estradiol differed between methods; ER-binding assay =1000 pM >> YES = 10 pM >ER-CALUX = 0.5 pM, respectively. Korner et al. (*74*) reported a detection limit of 0.3 ng estradiol L^{-1} for

an E-screen assay (proliferation assay of human estrogen receptor-positive MCF-7 breast cancer cells) used to detect estrogenic chemicals in municipal sewage treatment works.

In vivo methods provide more comprehensive information than in vitro tests about the ability of an estrogenic substance to induce a physiological response. Rodent uterotrophic assays have served as the standard in vivo estrogen analysis for many years (*125-128*). The utility of rodent assays for routine environmental analysis is limited, but the estrogenic activity of cow feces and poultry excreta has been measured using the approach (*129,130*). More recently, a variety of fish, reptile, and amphibian bioassays have been developed for monitoring the in vivo exposure of aquatic organisms to estrogenic substances (*131-137*). Vitellogenin production in fish has been widely used as a biomarker for the evaluation of estrogenic activity in municipal wastewater effluent (*138-141*).

Livestock Excretion

Steroidal estrogen hormones are excreted to the environment in the urine and feces of all species, sexes, and classes of farm animals (*142*). However, different estrogens are associated with different livestock species. Cattle (*Bos taurus*) excrete ≥90% of estrogens as 17α-estradiol, 17ß-estradiol, and estrone as free and conjugated metabolites (*43,143-147*). The 17α-estradiol epimer is much more prevalent than 17ß-estradiol. Conversely, 17α-estradiol rarely occurs in the excreta of swine (*Sus scrofa*), or poultry (*Gallus domesticus*) (*58,148,149*). They excrete 17ß-estradiol, estrone, and estriol plus conjugates (*58*). The α and ß stereochemical distinction of estradiol might be useful for identifying the livestock species contributing to waterway contamination (cattle vs poultry or swine), but this possibility has not been studied.

Different species also excrete estrogens by different routes. Radiotracer studies showed that cattle excrete estrogens mostly in feces (58%), whereas swine and poultry excrete estrogens mostly in urine (96% and 69%, respectively) (*145,148,150*). However, these ratios change during pregnancy (*144*). Since urine and feces are not usually handled separately in commercial animal production systems, the route of excretion would not appear to be an important environmental consideration (*142*). However, urinary estrogens are mostly conjugates, whereas fecal estrogens are excreted as unconjugated "free" steroids (*150*). At present, the environmental significance of conjugated vs. unconjugated estrogens is debatable due to a lack of information regarding conjugate fate (discussed later).

Estimates, calculated from literature values, of the estrogen excretion rates of cattle, swine, and poultry are given in Tables 2, 3, and 4, respectively. The various studies of urinary and fecal estrogen excretion were originally intended for describing the patterns of hormonal changes that occur during estrus and pregnancy with the practical purpose of establishing calibrated tests that could be used for fertility control or diagnosing pregnancy (*42-44,46,65,92,93,100,144,146,151-153*). The usefulness of the data for environmental purposes is limited because the data represent only sexually mature, female animals from a few breeds. Several factors (e.g., age, mass, diet, season, health status, circadian variation) may contribute to excretion rates and are not easily accounted for (*152*). Furthermore, few data were found which address estrogen excretion by sexually immature, sexually modified (ovariectomized, castrated), or male animals (*154,155*). The contribution of estrogens from these animals needs to be better resolved.

Another criticism of the excretion data is that ambiguous quantification methods

Table 2-2. Estimated rates of fecal and urinary estrogen excretion from cows.

Reproductive Stage	N	Excretion Rate/ 1000 kg LAM†	Estrogens Measured	Method	Reference
fecal excretion		($\mu g\ d^{-1}$)			
non-pregnant	21	600±200	E2α	RIA	(*43*)
non-pregnant	7	400±10	E1,E2α[‡],E2β	RIA	(*65*)
0-80 d pregnant	10	300±nd	E1,E2α,E2β	RIA	(*144*)
0-84 d pregnant	7	400±20	E1,E2α[‡],E2β	RIA	(*65*)
80-210 d pregnant	10	1500±nd	E1,E2α,E2β	RIA	(*144*)
140-200 d pregnant	7	11400±1200	E1,E2α[‡],E2β	RIA	(*65*)
210-240 d pregnant	10	5400±nd	E1,E2α,E2β	RIA	(*144*)
urinary excretion					
non-pregnant	7	500±40	E1,E2α[§],E2ß	RIA	(*147*)
55-81 d pregnant	5	700±60	E1,E2α[§],E2ß	RIA	(*147*)
101-123 d pregnant	13	14400±nd	E1,E2α,E2ß,E3	FL	(*83*)
111 d pregnant	3	34300±nd	E1,E2α,E2ß,E3	FL	(*156*)
107-145 d pregnant	4	3400±1200	E1,E2α[§],E2ß	RIA	(*147*)
165-175 d pregnant	5	28800±nd	E1,E2α,E2ß,E3	FL	(*83*)
205-209 d pregnant	4	22300±2500	E1,E2α[§],E2ß	RIA	(*147*)
250-254 d pregnant	5	86800±28000	E1,E2α,E2ß,E3	FL	(*83*)
271-285 d pregnant	13	163000±20000	E1,E2α,E2ß,E3	FL	(*83*)

† LAM– live animal mass; calculations based on typical animal weight of: 640 kg for dairy (*157*);‡ 11% 17α-estradiol cross-reactivity;§ 32% 17α-estradiol cross reactivity; N – number of animals, nd – no data, E1 – Estrone, E2 – Estradiol, E3 – Estriol, RIA– radioimmunoassay, FL– fluorimetry.

were used. As mentioned previously, colorimetric procedures lack sensitivity and selectivity for estrogens (*98*) and the enzyme immunoassay and radioimmunassay methods can suffer from false-positive interferences due to endogenous enzymes, matrix effects, and chromagens (*114,115*). Furthermore, complete estrogen profiles were rarely determined by any of the researchers. Thus, the data appear to be of insufficient quality for accurately calculating the total mass flux of estrogens to the environment from whole populations of cattle, swine, or poultry. Other researchers have not been so apprehensive. Lange et al. (*158*) calculated estrogen excretion for various livestock species. They reported that cattle, pigs, and chickens contribute 45, 0.8, and 2.7 Mg estrogens yr^{-1}, respectively, in the United States.

Table 2-3. Estimated rates of fecal and urinary estrogen excretion from sows.

Reproductive Stage	N	Excretion Rate/ 1000 kg LAM†	Estrogens Measured	Method	Reference
fecal excretion		($\mu g\ d^{-1}$)			
non-pregnant	4	800±nd	E1,E2ß,E3	RIA	(*159*)
non-pregnant	69	100±70	E1	EIA	(*46*)
non-pregnant	6	600±250	E1‡,E2α,E2ß,E3	RIA	(*42*)
non-pregnant	27	900±nd	not specified	RIA	(*44*)
14-34 d pregnant	6	1500±nd	E1,E2ß,E3	RIA	(*159*)
25-33 d pregnant	466	1000±680	E1	EIA	(*46*)
0-35 d pregnant	30	1600±nd	E1‡,E2α,E2ß,E3	RIA	(*42*)
urinary excretion					
non-pregnant	4	600±350	E1	FL	(*93*)
non-pregnant	2	500±600	E1	FL	(*100*)
non-pregnant	2	400±300	E1	FL	(*92*)
0-42 d pregnant	2	4400±6200	E1	CL	(*160*)
42-77 d pregnant	2	5000±6200	E1	CL	(*160*)
77-105 d pregnant	2	108000±106000	E1	CL	(*160*)

† LAM– live animal mass; calculations based on typical animal weight of 61 kg for swine (*157*);‡ 122% estrone, 30% 17α-estradiol, 100% 17ß-estradiol, 64% estriol cross reactivity, N – number of animals, nd – no data, E1 – Estrone, E2 – Estradiol, E3 – Estriol. RIA– radioimmunoassay, EIA– enzyme immunoassay, FL– fluorimetry, CL– colorimetry.

Table 2-4. Estimated rates of urinary estrogen excretion from non-laying and laying hen chickens.

Reproductive Stage	N	Excretion Rate/ 1000 kg LAM†	Estrogens Measured	Method	Reference
		($\mu g\ d^{-1}$)			
non-laying	3	600±30	E1	CL	(*90*)
non-laying	1	500±nd	E1,E3	CL	(*96*)
non-laying	1	400±20	E1	CL	(*90*)
non-laying	2	1400±550	E1,E2β	CL	(*95*)
non-laying	2	900±nd	E1,E3	CL	(*96*)
laying	1	1600±nd	E1,E2ß	FL	(*89*)
laying	1	2100±80	E1	CL	(*90*)
laying	1	2700±130	E1,E3	CL	(*96*)
laying	1	1400±50	E1	CL	(*90*)
laying	2	3500±430	E1,E2β	CL	(*95*)
laying	3	1600±nd	E1,E3	CL	(*96*)

† LAM– live animal mass; calculations based on typical animal weight of 1.8 kg for layers (*157*);N – number of animals, nd – no data, E1 – Estrone, E2 – Estradiol, E3 – Estriol, CL– colorimetry, FL– fluorimetry.

Another way of estimating the risk posed by manure-borne estrogens is to measure the concentrations of estrogens in livestock wastes that are land-applied as soil amendments. This approach takes into consideration the degradation of estrogens during storage and accounts for losses associated with manure handling and treatment practices.

However, extensive surveys of different animal production systems are required to establish approximate ranges of estrogens in livestock wastes. Few studies have characterized the estrogen profile of cattle, swine, or poultry wastes (Table 5). Concentrations of 17ß-estradiol in various dairy, swine, and poultry wastes range from below detectable limits (BDL) to 239±30 $\mu g\ kg^{-1}$, BDL to 1215±275 $\mu g\ kg^{-1}$, and 33±12 to 904 $\mu g\ kg^{-1}$, respectively (*3-6,161*). More characterization data are needed to determine which type of livestock wastes are most estrogenic and if manure treatment strategies are needed to reduce estrogen concentrations to environmentally acceptable levels.

Environmental Fate

Conjugate Hydrolysis

The fate of estrogen conjugates is not clearly known. It is often assumed that common fecal microorganisms such as *Eschericia coli* are capable of hydrolyzing estrogen conjugates via glucuronidase and sulfatase enzymes to unconjugated forms (*72*). This assumption appears valid for estrogen glucuronides but is questionable for estrogen sulfates since measurable concentrations ($ng\ L^{-1}$) of these conjugates have been reported in sewers, sewage treatment works, and river water (*46,162-164*). D'Ascenzo et al. (*163*) demonstrated that estrogen sulfates are slowly hydrolyzed in septic tank wastewater. After a 10 h lag-phase, half-lives of estradiol-3-sulfate and estrone-3-sulfate were approximately 2.5 d at 20° C. Estriol-3-sulfate was more stable, with a lag-phase of 70 h and half-life of 5 d.

Table 2-5. Concentrations of estradiol and estrone reported in various types of dairy, swine, and poultry wastes (dry weight basis).

Waste type	N	17α-Estradiol	17ß-Estradiol	Estrone	Method	Reference
Dairy		----------(μg kg^{-1} solids)----------				
press cake solids	1	139±7	BDL	426±78	GC-MS	(*6*)
dry-stack (semisolid)	36	603±358	236±216	349±339	GC-MS	(*161*)
dry-stack (solid)	24	289±207	113±67	203±176	GC-MS	(*161*)
holding ponds	48	370±59	239±30	543±269	GC-MS	(*161*)
Swine						
Finishing lagoon	48	BDL	BDL	1507±382	GC-MS	(*161*)
Finishing hoops	18	BDL	160±26	217±52	GC-MS	(*161*)
Farrowing lagoon	16	BDL	BDL	1295±168	GC-MS	(*161*)
Farrowing pit	32	890±120	1215±275	4728±427	GC-MS	(*161*)
Poultry						
Broiler litter	3	ND	33±12	ND	EIA	(*3*)
Broiler litter	3	ND	133±6	ND	EIA	(*4*)
Broiler litter (Al treat.)	3	ND	101±2	ND	EIA	(*4*)
Broiler litter	1	ND	904	ND	EIA	(*5*)
Broiler litter (females)	10	ND	65±7‡		RIA	(*165*)
Broiler litter (males)	10	ND	14±4‡		RIA	(*165*)
Layer litter	17	ND	533±40‡		RIA	(*165*)
Rooster litter	10	ND	93±13‡		RIA	(*165*)

‡ Reported as 17ß-estradiol plus estrone; N–Number of samples BDL, below detectable limits; ND; not determined; GC-MS, gas chromatography-mass spectrometry; EIA, enzyme immunoassay; RIA, radioimmunoassay.

Few studies have evaluated the stability of conjugated estrogens in manure. Vos (*159*) incubated ^{3}H-estrone-sulfate and ^{3}H-estrone-glucuronide with sow feces ≤30 min at 20°C. Estrone glucuronide was rapidly deconjugated (90% in 30 min) in the fecal suspension, but estrone sulfate was not hydrolyzed. Raman et al. (*6*) incubated dairy waste with *Helix pomatia* glucuronidase-sulfatase to hydrolyze conjugated estrogens. No differences in free estrogen concentrations were found between hydrolyzed and non-hydrolyzed samples. These results suggested that estrogen sulfates were not present in the dairy waste (*6*), but it is not clear if the limitations of the enzyme hydrolysis (discussed previously) was considered in their assessment.

Degradation of Unconjugated Estrogens

The biodegradation and transformation of unconjugated estrogens has been studied in soil, water, and manure for several years. In 1947, Turfitt (*166*) examined the biodegradation of 17α-estradiol and estrone using 355 different cultures of bacteria isolated from five different soil types. No culturable bacteria were found in loam, marl, or alkaline peat soils that could metabolize estradiol. However, one *Proactinomyces spp.* was isolated from an acid sand and two strains were found in arable soil that could use estradiol as a carbon source. Estrone was degradable by one species of *Proactinomyces spp.* in the arable soil, but no degradation was observed with organisms from the other four soils.

Stumm-Zollinger and Fair (*167*) reported that bacteria (*Pseudomonas*) living in soils and wastewater can use natural estrogens as carbon sources. When high concentrations (300 mg L^{-1}) of both estradiol and estrone were provided in the growth medium, substrate elimination was only 10 to 15% during 2 wk of incubation. However, when estrogen concentrations were reduced to 20 mg L^{-1} (near solubility limits),

estrogens disappeared within 10 d and there was no evidence of steroid-like metabolites remaining in the culture solution. Tabak and Bunch (*69*) evaluated activated sewage sludge, primary settled sewage, and soil as sources of microorganisms capable of degrading estrogens. Activated sludge and soil were better sources of estrogen decomposers than the primary settled sewage. Additional experiments by Tabak and Bunch (*69*) using activated sludge as an innoculum showed that 86 to 100% of estrogens were eliminated from the culture solution within 4 wk of incubation.

Recently, Colucci et al. (*64*) studied the dissipation (decrease in extractable/ bioavailable concentrations and mineralization) of ^{14}C-17ß-estradiol in loam, sandy loam, and silt loam soils from Canada. The biological activity (determined by a yeast assay) of estradiol was rapidly dissipated in all soils, and 17ß-estradiol was rapidly converted to estrone. The accumulation of estrone in the loam soil was maximal at 6 h, but was undetectable thereafter. In the silt loam and sandy loam soils, however, estrone was detectable for 3 months. Autoclaving the soils did not prevent the oxidation of estradiol to estrone. This result suggests that either there was an incomplete sterilization of the soil, the enzyme responsible for estradiol transformation survived autoclaving, or that estradiol oxidation can proceed abiotically. The mineralization (cleavage of the phenolic ring) of the estradiol in the soils tested was relatively slow compared with the rates of dissipation; only 12 to 17% of added ^{14}C-17ß-estradiol was evolved as $^{14}CO_2$ after 3 months of incubation at 30°C. The highest rates of mineralization were observed in the sandy loam soil and the lowest rates were observed in the silt loam soil. A comparison of soil pH, organic matter content, and texture did not reveal any consistent effect of these soil properties. When the soil temperature was increased from 4 to 37°C, mineralization

in the loam soil increased from 4 to 15% after 61 days of incubation. Mineralization also increased from <1 to 20% after 73 d of incubation when the moisture content of the sandy loam soil was increased from air-dry to 15%. However, when moisture content of the same soil was increased to field capacity (24%), the amount of estradiol mineralized decreased sharply to 8%. The authors concluded that estrogens are biodegradable in soils by ubiquitous microorganisms that require no prior adaptation (*64*).

Rapid biodegradation of estrogens in river water was reported by Jurgens et al. (*168*). The half-lives of estradiol and estrone at 20° C ranged from 0.2 to 9 d and from 0.1 to 11 d, respectively. No significant losses of estradiol were found in sterile controls. Lai et al. (*169*) reported that common freshwater algae (*Chlorella vulgaris*) are capable of oxidizing 17ß-estradiol to estrone.

Jarvenpaa et al. (*37*) found that aerobic and anaerobic microflora isolated from the human intestinal tract and human feces were capable of transforming estrogens during 24 to 72 h incubation. *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*, converted estradiol to estrone, and vice versa. *Streptococcus faecalis* (four strains) oxidized estradiol to estrone, and one strain transformed estrone to 16α-hydroxyestrone. *Bacteroides fragilis* reduced estrone to estradiol, but also converted estrone to 16α-hydroxyestrone. *Staphylococcus aureus* and *M. smegmatis* reduced 16α-hydroxyestrone to estriol. *Candida albicans*, *Enterobacter cloacae*, *E. coli* (two strains), *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Proteus vulgaris* were unable to metabolize estrone, estradiol, or 16α-hydroxyestrone to any other products (*37*).

Shore et al. (*165*) incubated broiler litter for 1 week at different pH values, with and without the addition of antibiotics (penicillin/streptomycin), and found significant reductions in estrogen concentrations at pH 5 and 7, but no change at pH 1 or 12. When antibiotics were added to the litter, estrogens persisted. Schlenker et al. (*170*) studied the degradation of estrogens in cattle feces by incubating manure samples for 12 weeks at 20 to 23°C. The median concentrations of total estrogens extracted from the manure were unchanged for 9 weeks, but were reduced by 80% after 12 weeks. Schlenker et al. (*171*) tested *E. coli* and *Clostridium perfringens* for their ability to degrade fecal estrone in cow manure. The *E. coli* had no effect on estrone concentrations, but the *C. perfringens* reduced the average concentration of estrone from ~16 $\mu g\ L^{-1}$ to ~11 $\mu g\ L^{-1}$ during the 48 h incubation. Schlenker et al. (*172*) evaluated the influence of temperature on the stability of estrogens in the feces of cows. At 5°C, the median concentrations of total estrogens extracted from the manure fell below initial concentrations after 12 weeks of incubation. At 30°C, however, estrogen was almost completely eliminated from the samples within 3 weeks. Similar studies of estrogen degradation in dairy cattle manure were done by Raman et al. (*6*). Press cake samples were spiked with 17ß-estradiol and incubated at temperatures ranging from 5 to 50°C. The effects of acidification on estrogen transformation and degradation during sample storage were also evaluated. At all temperatures, estradiol concentrations rapidly declined during the first 24 hours of incubation, and estrone accumulated. Total estrogen removal rates followed the pattern of estrone degradation, and these data were fitted to a first-order decay model. Rate constants increased from ~0.03 d^{-1} at 5°C to ~0.12 d^{-1} at 50°C. Acidification to pH 2 reduced rates of estrogen transformations at both 5 and 30°C, but a 15 and 31% loss,

respectively, of total estrogen was still observed when samples were stored for 7 days. The authors speculated that *Cornybacterium spp.* were partially responsible for the estrogen transformations in their study (*6*).

Based on the data available, it appears that estrogens are biodegraded in the environment by many different types of microorganisms. Few degradation mechanisms have been proposed, but the oxidation of estradiol (C-17 alcohol) to estrone (C-17 ketone) is frequently reported (*6,29,30,64,162*). It can be hypothesized that the reaction is catalyzed by bacterial or fungal dehydrogenases (*173-176*). Further degradation of estrone may involve C-2 or C-4 hydroxylation of the phenolic A-ring and subsequent ring cleavage and/or C-16 hydroxylation of the D-ring (*37,38,177*). The phenoloxidase group of enzymes (e.g., laccase, tyrosinase, and peroxidase) that are produced by bacteria, white-rot fungi, and plants might be critical for the degradation process (*178-180*). If so, the phenolic estrogens may be oxidized to quinones, which may polymerize into humus-like macromolecules (*39,181-192*). Recently, Suzuki et al. (*193*) reported that ligninolytic enzymes (manganese peroxidase and the laccase-mediator system with 1-hydroxybenzotriazole as mediator) removed ≥80% the estrogenic activity of 17ß-estradiol during a 1 h laboratory incubation.

If estrogens behave like other phenolic compounds in the environment, they may also oxidize abiotically. For example, Lehmann et al. (*194*) demonstrated that the oxidation of phenolic acids in soils can be coupled with the reduction of Fe and Mn oxides. The catalytic effects of Mn (IV), Fe(III), aluminum, and silicon oxides on the formation of phenolic polymers in soils was studied by Shindo and Huang (*195*). Mn oxides caused phenolic compounds to be converted to humic acid with a high degree of

humication via oxidative polymerization. Mn oxide reduction is an important mechanism in the oxidation of phenols in aquatic systems (*196*). No literature was identified which have specifically examined the role of Mn in the environmental fate of estrogens.

Sorption and Mobility

Estrogens are nonvolatile, slightly hydrophobic compounds that do not ionize at normal environmental pH, and should be extensively sorbed by aquatic sediments and soils. Holthaus et al. (*31*) studied the sorption of 17ß-estradiol to river sediments. They reported sorption coefficients (K_d) that ranged from 4 to 74 L kg^{-1} for bed sediments and from 21 to 122 L kg^{-1} for suspended sediments. Casey et al. (*197*) reported that ^{14}C-17ß-estradiol is strongly sorbed by soils. Sorption coefficients (K_d) ranged from 86 to 6670 L kg^{-1} as determined by batch equilibrium studies with four Mollisols. Positive correlations were found between estradiol sorption and silt content (r^2=0.92) and organic carbon (r^2=0.62). Column experiments demonstrated that estradiol is not easily leached through the soil. Lee et al. (*198*) reported that hydrophobic partitioning is the dominant mechanism for the sorption of 17ß-estradiol and estradiol metabolites to soil. They reported that K_d values in two soils ranged from ~3.6 to 83 L kg^{-1}, but Log K_{oc} values were ~3.2 and 3.5, respectively (*198*). Colucci et al. (*64*) also reported a strong retention of estrogens to soil particles. Within 3 days of contact between ^{14}C-estradiol and loam, sandy loam, and silt loam soils, 91, 70, and 56% of the radioactivity, respectively, were nonextractable from the soils using ethyl acetate or acetone. Variations in soil properties (soil pH 5.8 to 7.4, organic matter 0.8 to 3.2%) were not consistently related to sorption capacity. However, when soils were autoclaved, the amount of extractable radioactivity remained constant for several days. Their results suggested that the formation of nonextractable (bound) residues in the soils was microbially mediated (*64*). Colucci and

Topp (*199*) concluded that estrogen dissipation via the formation of soil-bound residues greatly reduces the risk of contamination of water adjacent to agricultural soils treated with municipal biosolids or livestock wastes.

Though laboratory-based experiments have suggested that ^{14}C estrogens are rapidly sorbed by soil particles, it should be recognized that sorption was evaluated without additions of manure. The information thus gained does not allow assessment of the effects of the chemical, physical, and microbiological changes that can occur in a soil following a manure application. It can be speculated that natural surfactants and colloids might increase the mobility of estrogens in soils and together with erosion and preferential flow mechanisms could lead to the transport of manure-borne estrogens to waterways.

Occurrence in Manure-Impacted Water

Field studies with manure have demonstrated that estrogens are sufficiently mobile to impact surface and groundwater quality. For example, Shore et al. (*8*) surveyed estrogen (17ß-estradiol plus estrone) concentrations in a few small streams, an irrigation pond, and a farm well impacted by the land application of poultry litter (no estrogen concentrations reported or application rates specified). Estrogen concentrations in the streams increased from <0.5 ng L^{-1} to 5 ng L^{-1} following poultry litter application, whereas concentrations in the pond decreased from 23 to 5 ng L^{-1} during the study period (9 months). Low concentrations (< 0.1 ng L^{-1}) of estrogens were found in the well water samples.

Nichols et al. (*4*) tested the hypothesis that land-applied poultry litter contributes 17ß-estradiol to runoff water. They reported that the water-soluble 17ß-estradiol contents of normal and alum treated litter were 133 and 102 µg kg^{-1} (dry-weight basis),

respectively. Estradiol concentrations in the runoff water increased with litter application rate (1.76 to 7.05 Mg ha^{-1}) for both untreated and aluminum sulfate treated amendments. A maximum concentration of 1280 ng estradiol L^{-1} was detected in first-storm runoff water from plots amended with normal poultry litter. Aluminum sulfate treatment of the litter significantly reduced 17ß-estradiol concentrations in first-storm runoff by 42%, presumably due to the flocculation of soluble organic compounds with aluminum. An additional study by these authors compared the effectiveness of varying lengths of grass filter strips to help reduce concentrations of 17ß-estradiol in runoff water from fescue-applied poultry litter (*5*). The water-extractable 17ß-estradiol concentration of the litter sample was 904 µg kg^{-1}. The litter application rate of 5 Mg ha^{-1} was consistent with the recommendation for tall fescue in Arkansas. Concentrations of 17ß-estradiol in runoff from plots without a grass filter (controls) averaged 3500 ng L^{-1}. Compared with the control plots, estradiol concentrations were reduced by 58, 81, and 94% after transport through 6.1, 12.2, and 18.3 m long grass filters, respectively. Bushee et al. (*1*), investigated runoff concentrations of 17ß-estradiol from plots amended with horse bedding or municipal sludge. The horse bedding and municipal sludge contained 35 µg kg^{-1} and 5 µg kg^{-1} (author did not indicate wet or dry-weight basis) of 17ß-estradiol, respectively. The horse bedding was applied to fescue grass plots at a rate of 9.1 Mg ha^{-1} and the sludge at a rate of 7.7 Mg ha^{-1}. The cumulative transport of estradiol from the plots after 30 min. of simulated rainfall was 70 and 12 mg ha^{-1} for horse bedding and municipal sludge, respectively. In contrast to the findings of Nichols et al. (*4*), alum treatment of either material did not significantly reduce estradiol losses.

Finlay-Moore et al. (*3*) measured 17ß-estradiol concentrations in runoff and soil from grazed and ungrazed pastures fertilized with broiler litter. The ethyl acetate extractable concentrations of 17ß-estradiol in three poultry litter samples ranged from 20 to 35 µg kg (dry weight basis). After litter was applied, concentrations of 17ß-estradiol in runoff were 20 to 2530 ng L^{-1}, depending on litter application rate and time between application and runoff. High background estradiol concentrations were found in runoff, ranging from 50 to 150 ng L^{-1}. Prior to the addition of litter, the concentration of 17ß-estradiol in the soil was ~55 ng kg^{-1}. Immediately following the application of litter, elevated levels of 17ß-estradiol were detected (≤675 ng kg^{-1}). The high concentrations did not persist in surface (upper 2.5 cm) soil for more than a few weeks. No samples were collected from lower soil depths, so leaching of estradiol into the soil profile or degradation in the soil could not be determined. There were no significant effects of grazing cattle on the concentrations of 17ß-estradiol in the runoff (*3*).

Dyer et al. (*2*) measured 17ß-estradiol concentrations in runoff from bermudagrass plots fertilized with liquid dairy manure. They applied manure containing 3300 ng L^{-1} (wet weight basis) of 17ß-estradiol to plots at rates equivalent to 0, 65, and 142 kg N ha^{-1}. Runoff samples were collected from the plots following natural rainfall events (rainfall dates or amounts not reported). Estradiol concentrations from control plots ranged from below detectable limits (1.6 ng L^{-1}) to 2.1 ng L^{-1}. At the highest rate of manure application, estradiol concentrations reached 41 ng L^{-1}, but decreased steadily to background (control) concentrations by the end of the study (3 months). These results suggested that N-based application rates of dairy manure could potentially increase 17ß-estradiol concentrations in runoff.

Nationwide reconnaissance data by the U.S. Geological Survey showed estradiol and estrone concentrations ≤200 and ≤112 ng L^{-1}, respectively, in a network of 139 streams in 30 states impacted by animal wastes (*200*). Peterson et al. (*201*) sampled five springs from the mantled karst aquifer system of northwest Arkansas (a major poultry and cattle production region) for fecal coliforms and 17ß-estradiol. Concentrations of 17ß-estradiol ranged from 6 to 66 ng L^{-1}. At all locations, there was a positive correlation between estradiol concentrations and the concentrations of both fecal coliform (r^2 ranging from 0.49 to 0.86) and *E. coli* (r^2 ranging from 0.40 to 0.88), suggesting that estradiol and bacteria were moving through the aquifer system in a similar fashion. The authors concluded that estradiol of livestock origin was directly affecting the groundwater quality of the springs.

The concentrations of 17ß-estradiol reported in the abovementioned studies of surface and groundwater warrant careful attention due to the previously stated 10—100 ng L^{-1} range of biological significance for aquatic organisms. It should be noted that, all of the field studies, except for Kolpin et al. (*200*), determined 17ß-estradiol using immunoassay. The authors provided few quality control details (besides manufacturer's statements) regarding the sensitivity, accuracy, precision, and reliability of the analytical methods used. As previously stated, immunoassays can be affected by a number of interferences, especially when chromatographic purification is not performed. Surface water is known to contain natural organic matter that can interfere with immunoassays in a manner that causes false positive signals (*73*). Therefore, the reported runoff concentrations may be overestimated. If not, the contamination of surface and groundwater by manure was probably worse than predicted by the evaluation of 17ß-

estradiol alone due to the unmeasured contribution of estrone and other estrogens. In either case, the validation of immunoassay results by the use of nonambiguous quantification methods such as LC-MS or GC-MS would add credibility to the measured estrogen concentrations.

Synthesis

Estrogen contamination of the environment is of concern because there is evidence that low part per trillion (10—100 ng L^{-1}) concentrations of these chemicals can adversely affect the reproductive biology of vertebrate species by disrupting the normal function of their endocrine systems. Livestock wastes are a potential source of estrogens to the environment via direct excretion in urine and feces or via land-application of manure. At this time, insufficient characterization data exist to quantify the potential mass flux of estrogens to the environment from livestock populations or manure. Based on the low water solubility and hydrophobic properties of estradiol, estrone, and estriol, sorption to organic matter and subsequent transformation and biodegradation pathways are likely removal mechanisms for these compounds. Laboratory-based studies with estrogens added in pure chemical form have generally supported a rapid dissipation hypothesis. However, field studies with land-applied manure have not strictly followed these principles. Significant concentrations of 17ß-estradiol have been noted in manure, manure-impacted soil, manure-impacted runoff, and manure-impacted groundwater.

There are several issues that need to be addressed regarding the lack of agreement between laboratory and field studies. First, the laboratory studies of sorption and persistence have tested these parameters by the addition of estrogens into the soil and water systems without additions of manure. The information thus gained does not allow assessment of the possible effects of the profound chemical, physical, and

microbiological changes that can occur in a soil following a manure application. Estrogens in manure may be bound to the organic substances in a way that protects them from degradation. Hydrophobic estrogens may also sorb to hydrophobic parts of organic molecules that are otherwise hydrophilic (natural surfactants) or be associated with colloidal fractions. Preferential flow of water through the soil may also increase estrogen transport. Perhaps these (or other) mechanisms can account for the apparent mobility of estrogens in soils and their presence in waterways.

Conversely, the field studies have frequently used immunoassay techniques to quantify the concentrations of estrogens in the manure, soil, and water samples. Unfortunately, few details have been provided by any of the authors regarding the sensitivity, accuracy, and reliability of the analytical methods used and no specific purification protocols have been specified prior to the quantification step. Based on the various types of interferences that can occur with immunoassays, the methods may have overestimated the hormone concentrations. On the other hand, if the immunoassay results are accurate, then it seems likely that the contamination of the surface and groundwater was probably worse than predicted by the evaluation of 17ß-estradiol alone due to the unmeasured contribution of other estrogens in the samples. The validation of immunassay results by the use of additional quantification techniques like LC-MS or GC-MS would add credibility to the measured hormone concentrations. In vitro methods like the YES assay might be useful for the estimation of estrogenicity, but these techniques should be extensively validated to ensure that soil, manure, and water samples do not contain cytotoxins, endogenous enzymes, or other substances that can interfere with the quantification.

Critical Research Needs

In light of the information presented in this review, a number of research priorities can be suggested: (i) There is a critical need to use standardized methods for the analysis of estrogens in manure, soil, and water. Juridical proof of estrogen contamination will require LC-MS or GC-MS quantification methods. (ii) More national, state, and local surveys of manure-impacted surface and groundwater resources need to be conducted to determine if estrogen contamination is a widespread phenomenon or is localized to intensive livestock production areas. Other water quality indicators (e.g., fecal coliforms, nitrates, phosphorus) should also be measured during these surveys so that maximum information can be gained about any estrogen pollution attributable to manure. Wildlife exposed to estrogen-contaminated waterways and/or test organisms should be studied for evidence of reproductive abnormalities. (iii) More information is needed about the types and amounts of estrogens that exist in fresh livestock excreta (urine and feces) and manure. Characterization experiments should be broad in scope to reflect a wide range of livestock production techniques and manure handling and storage practices. Better estimates of the total mass flux of estrogens to the environment could therefore be made. (iv) More work needs to be done regarding the fate of conjugated (especially estrogen sulfates) and unconjugated estrogens in manure, soil, and water. The rates of deconjugation reactions, the oxidation/reduction relationship between estradiol and estrone, and the kinetics of biodegradation should be measured in the various matrices. Experiments that reveal the influence of temperature, moisture, pH, and microbial activity would also improve knowledge of estrogen persistence under various environmental conditions. Ideally, the specific enzyme(s) and/or soil mineral(s) participating in estrogen transformation and mineralization reactions should be identified

so that degradation and sorption mechanisms can be proposed. Partitioning experiments need to identify the surfaces responsible for estrogen sorption (organic matter, Fe and Al oxides, etc.) and the chemical conditions (pH, salinity, etc.) that enhance binding of estrogens to solid phases in manure, soils, and aquatic systems. Desorption kinetics and aging phenomena should also be evaluated because estrogens may form nonextractable (bound) residues in soils. More field and laboratory studies are needed to determine the mechanisms of estrogen transport (surface runoff vs. leaching) to waterways. (v) Besides estrogens, other hormonally active agents in manure (e.g., androgens, gestagens, growth promoters, antibiotics, phytoestrogens) need to be characterized and studied. Ultimately, it may be necessary to develop cost-effective manure treatment strategies to reduce or eliminate manure-borne endocrine disruption hazards.

CHAPTER 3
COMPARISON OF THREE ENZYME IMMUNOASSAYS FOR MEASURING 17ß-ESTRADIOL IN FLUSHED DAIRY MANURE WASTEWATER

Introduction

Dairy farms in the United States generate ~21.5 million metric tons of recoverable manure solids each year that must be managed in a way that does not adversely impact the environment (*202*). Typically, dairy wastes are applied to nearby pasture and croplands as soil amendments because they contain various plant nutrients, including N, P, and K. However, recent literature indicates that agricultural drainage waters may become contaminated with natural steroidal estrogen hormones such as 17ß-estradiol when livestock wastes are land applied (*1-5,8*).

Estrogen contamination of waterways is a concern because low concentrations (10—100 ng L^{-1}) of these chemicals in water can adversely affect the reproductive biology of vertebrate species such as fish, turtles and frogs by disrupting the normal function of their endocrine systems (*9-13*). For example, 17ß-estradiol concentrations ≥30 ng L^{-1} induced vitellogenin (an egg yolk precursor protein that is normally produced only by adult females) synthesis and abnormal testicular growth in male fathead minnows (*Pimephales promelas*) after 21 days of laboratory exposure (*12*). However, research evaluating the in situ effects of manure-borne estrogens on wildlife is limited. Irwin et al. (13) reported that vitellogenin production by female painted turtles (*Chrysemys picta*) in ponds was significantly affected by estrogens in beef cattle runoff compared with turtles in ponds unexposed to beef cattle runoff.

Clearly, it is important to have accurate information about the occurrence of estrogens in manure so that any estrogen contamination of waterways resulting from dairy waste disposal can be prevented or minimized. Estrogen characterization of dairy wastes is not a trivial task, however, due to the low concentrations that must be measured, the difficulties associated with extracting estrogens from manure, the chemical complexity of the resulting extract matrix, and the potential for degradation losses to occur during sample storage (*6*). A variety of quantitative enzyme immunoassays (EIA) have been used for the determination of 17ß-estradiol in manure-impacted surface and groundwater and in livestock wastes (*1,3,4,201*). The popularity of EIA for 17ß-estradiol analysis is attributable to widespread commercial availability, ease of use, pg mL^{-1} detection limits, and a lack of alternative quantitation methods. However, a variety of interferences arising from poor standardization, cross reactivity, and matrix effects associated with protein binding, humic substances, and endogenous enzymes and chromagens, can adversely affect the quality (accuracy, precision, reproducibility) of the data generated (*73,112-114*). Thus, depending on sample complexity and EIA reagents, antibodies, and protocol, a potential exists for different EIA systems to yield dissimilar and/or inaccurate results. The objective of this study was to determine if three different commercially available 17ß-estradiol EIAs yielded similar estimates of the endogenous concentration of 17ß-estradiol in flushed dairy manure wastewater.

Materials and Methods

Sample Collection

Many dairies use hydraulic flushing for manure management, followed by primary treatment (mechanical screening or sedimentation, or both) to remove coarse solids. The liquid fraction of flushed dairy manure after settleable solids are removed is referred to as

flushed dairy manure wastewater (FDMW) (*203*). A bulk grab sample (1 L) of FDMW was collected from the University of Florida Dairy Research Unit located at Hague, FL and was transported to the laboratory in less than 1 h for liquid-liquid ether extraction. Two weeks later, a second 1 L sample of FDMW was collected and processed in a similar manner. The total solids content of these samples was determined by a standard method (*204*). The first and second FDMW samples contained an average of 0.57 and 0.62% total solids, respectively.

Ether Extraction

For each wastewater sample, four 20-mL aliquots of FDMW were poured into separate 50 mL glass centrifuge tubes. Twenty mL of pesticide grade ethyl ether (Fisher Scientific, Pittsburgh, PA) was added to each tube for extraction of 17ß-estradiol. Liquid-liquid extraction with ether was used for sample preparation because it is a traditional solvent of choice for steroid extraction from biological samples; ether extraction is recommended for sample purification by the EIA manufacturers used in this study, and it has been used previously for extraction and purification of dairy waste samples for EIA analysis (*6*).

The tubes were shaken horizontally for 2 h followed by centrifugation at 500 *g* for 5 min to facilitate layer separation. Three 4 mL aliquots (one for each assay) of the ether extract were subsampled from each tube and placed into separate 5 mL evaporation flasks. The ether was evaporated to dryness at 40°C under N_2. The dried sample was immediately reconstituted in 1 mL of bulk assay buffer that was purchased from each immunoassay manufacturer. The reconstituted samples were individually sonicated for ~1 min. to enhance solubilization in the assay buffer. The samples were poured into 1.5

mL micro centrifuge tubes, capped tightly, and stored overnight at -20 °C prior to immunoassay analysis.

Immunoassay Description

Enzyme immunoassay kits for the quantitative determination of 17ß-estradiol were purchased from Assay Design, Inc. (cat. no. 900-008; Ann Arbor, MI), Diagnostics Systems Laboratories, Inc. (cat. no. DSL-10-4300; Webster, TX), and Immuno-Biological Laboratories, Inc. (cat. no. RE 52041; Minneapolis, MN). The immunoassay kits were designated A1, A2, and A3, respectively. The A1 immunoassay (catalog no. 900-008) was selected because it has been used previously for the quantification of 17ß-estradiol in dairy wastes (*6*). The A2 and A3 immunoassays were selected based on their use of rabbit polyclonal antibodies (RPA), and the competitive assay principle, and a low cross reactivity with other steroids (Table 1).

Each of the EIAs used in this study were based on the competitive binding principle, whereby 17ß-estradiol and a fixed amount of enzyme labeled-estradiol compete for RPA binding sites. However, the A2 and A3 assays use RPAs that are directly coated onto the microplate wells, whereas the A1 microplate wells are coated with goat anti-rabbit IgG to capture the 17ß-estradiol-RPA complex. The alkaline phosphatase, streptavidin-horseradish peroxidase, and horseradish peroxidase enzyme tracers used by A1, A2, and A3, respectively, represent commonly-used enzyme reagents for estrogen immunoassay (Table 1) (*159,205-207*). As shown in Table 1, each immunoassay has low (<5%) cross reactivity with other estrogen steroids.

Table 3-1. Description and cross reactivity of three enzyme immunoassay systems used for measuring 17ß-estradiol in flushed dairy manure wastewater.

Description	A1	A2	A3
Assay principle	Competitive	Competitive	Competitive
17ß-Estradiol antibody	rabbit polyclonal	rabbit polyclonal	rabbit polyclonal
Matrix	TBS†	Serum	Serum
Conjugate/Enzyme	E2-ALP	E2-Biotin/SHRP	E2-HRP
Substrate	p-NPP	TMB	TMB
Range (pg mL^{-1})	0-30,000	0-6,000	0-2,000
MDL (pg mL^{-1})	29	7	10
Precision (CV%)	9	4	4
Cross-reactivity (%)			
17ß-Estradiol	100	100	100
17α-Estradiol	0.1	0.3	0.3
Estrone	4.6	1.4	2.1
Estriol	0.5	1.1	1.5

†TBS, Tris-buffered saline containing proteins and detergents and sodium azide as a preservative; E2, 17ß-estradiol; ALP, alkaline phosphatase; SHRP, streptavidin horseradish peroxidase; HRP, horseradish peroxidase; p-NPP, p-nitrophenol phosphate; TMB, tetramethylbenzidine; MDL, minimum detection limit.

Immunoassay Analysis

Each assay was performed according to the manufacturer's instructions. All standards and samples were assayed in duplicate and an average value was used to generate standard curves and interpolate unknown sample concentrations. Microplate washing was performed with an $EL_x50/8$ strip washer (Bio-Tek Instruments, Inc., Winooski, VT) using the wash buffer reagents provided by each company. The absorbance values of each well were measured using an FL 600 microplate reader (Bio-Tek Instruments, Inc.). A four-parameter logistic equation was used for all calibration curves (*208*).

Immunoassay performance characteristics including sensitivity, standardization, precision, and recovery of diluted and spiked samples were evaluated on both days of wastewater analysis. Sensitivity is defined as the lowest measurable concentration of 17ß-

estradiol that can be distinguished from the respective 0 pg mL^{-1} calibrator (95% confidence interval) associated with each EIA (*209*). Sensitivity was calculated for each EIA by interpolation of the mean of eight replicate samples of the respective 0 pg mL^{-1} calibrator minus two standard deviations.

Standardization accuracy refers to the ability of each EIA to yield a correct measurement of 17ß-estradiol for a known standard concentration. Standardization accuracy was evaluated at three concentrations (1500, 750, and 375 pg mL^{-1}) by diluting a 300,000 pg 17ß-estradiol mL^{-1} buffer solution (Assay Design Inc., Ann Arbor, MI), with the respective 0 pg mL^{-1} calibrator of each EIA. Three concentrations were measured to ensure accurate recovery at different interpolation points along the calibration curve. A recovery percentage for each standard concentration was calculated by dividing the measured sample concentration by the known sample concentration and multiplying the result by 100. The three resulting values were averaged to express EIA standardization accuracy.

Intra-assay precision refers to the within-run reproducibility of the 17ß-estradiol signal that is produced for a particular sample in an EIA. Precision was evaluated by calculating the percent coefficient of variation (CV%) observed between duplicate measurements corresponding to the four neat wastewater samples. The four resulting CV% values were averaged to express precision.

Recovery of diluted and spiked samples is a gauge of the linear relationship between 17ß-estradiol measured in diluted or spiked samples relative to the neat samples. Dilution recovery was measured by diluting each of the four neat wastewater samples with an equal volume of the respective 0 pg mL^{-1} calibrator of each assay. Spiked

recovery was measured by spiking the neat wastewater samples with an equal volume of the second greatest respective 17ß-estradiol calibrator from each EIA (i.e. A1, 7500 pg mL^{-1}; A2, 2000 pg mL^{-1}; A3, 1000 pg mL^{-1}). The second greatest calibrators were used for spiking to ensure that the resulting spiked sample concentrations would be interpolated from the mid-portion of the calibration curve of each assay. Dilution and spiked recovery was expressed as a percentage by dividing the measured concentration of the diluted or spiked sample by the theoretically expected concentration of the diluted or spiked sample, and the result was multiplied by 100.

Data Analysis

The experimental design was a two-way factorial (three immunoassay methods X two FDMW samples) with four replications. Experimental data were analyzed using the General Linear Model program of SAS with a separation of sample means by Duncan's multiple range test (*210*).

Results and Discussion

A summary of the immunoassay performance characteristics from each FDMW analysis is shown in Table 2. The measured sensitivity data corresponding to the first wastewater sample were similar to or better than the manufacturer's data for each EIA. However, the sensitivity data corresponding to the second analysis were three to four times larger for each assay. The average EIA sensitivity for both analyses was 62, 14, and 26 pg mL^{-1}, for the A1, A2, and A3 assays, respectively. The sensitivity data demonstrate the exceptionally low 17ß-estradiol concentrations that can be measured using EIA.

Recovery data shown in Table 2 demonstrates that the A1 and A2 assays were relatively well standardized for both analyses. The calibration of the A3 assay appeared to be somewhat less accurate for each individual analysis since it overestimated by 36%

and underestimated by 25%, respectively, the standard concentrations for the first and second analysis. Overall, however, the average recovery for both analyses was 105, 98, and 106% for the A1, A2, and A3 immunoassays, respectively. Therefore, it seems that each of the EIAs was reasonably well standardized.

Each assay also showed a high degree of intra-assay precision between duplicate samples. The CV% for both analyses averaged 8, 7, and 9%, respectively, for the A1, A2, and A3 assays. The low CV% values indicate that the chemical reactions involved in generating the 17ß-estradiol signals for each EIA were highly reproducible within the analytical run.

Table 3-2. Summary of performance data for analysis of two flushed dairy manure wastewater samples by three different immunoassays.

Performance characteristic	FDMW	n	A1	A2	A3
Sensitivity (pg mL^{-1})	1	8	25	7	10
	2	8	98	20	41
Standardization accuracy (%)	1	3	102	88	136
	2	3	108	108	75
Precision of replicate samples (CV%)	1	4	13	9	11
	2	4	3	4	7
Recovery of diluted samples (%)	1	4	92	109	124
	2	4	66	128	124
Recovery of spiked samples (%)	1	4	88	101	96
	2	4	96	89	85

n= number of samples; CV= coefficient of variation

Recovery of diluted samples ranged from 66 to 128%, depending on the EIA and day of analysis (Table 2). The recovery of diluted samples for both analyses averaged 79, 119, and 124%, respectively, for the A1, A2, and A3 assays. In contrast to diluted samples, recovery improved markedly when the neat samples were spiked with 17ß-estradiol. Recovery of the spiked samples averaged 92, 95, and 91%, respectively, for the A1, A2, and A3 immunoassays. Overall, the recovery of diluted and spiked samples

demonstrated a reasonably linear recovery of 17ß-estradiol at the different interpolation points evaluated from the standard curve.

Although some minor differences were encountered between assays regarding standardization accuracy, intra-assay precision, and recovery of diluted and spiked samples, the measured concentration of 17ß-estradiol in both sets of FDMW samples differed according to the EIA used (Fig. 1). The A1 assay consistently measured the greatest 17ß-estradiol concentrations and the A2 assay measured the lowest.

Because no differences were observed between EIAs when a pure solution of 17ß-estradiol was analyzed (standardization accuracy) (Table 2), the apparent difference between assays suggests that an interference affected 17ß-estradiol quantitation in FDMW samples in one or more of the EIAs. A known source of interference with the EIAs is the presence of other steroidal estrogens that are listed as crossreactants in Table 1. It was noticed that the apparent concentrations of 17ß-estradiol in the wastewater followed in the same qualitative order (A1>A3>A2) as the reported estrone cross reactivity of the different assays. Consequently, estrone was a suspected source of bias between assays. Hence, we measured estrone with an estrone EIA (catalog no. DB 520 51; Immuno-Biological Laboratories, Inc., Minneapolis, MN). Similar estrone EIAs were not available from the other companies for comparison. Estrone concentrations were 562 and 781 ng L^{-1} in the first and second wastewater samples, respectively. Based on the cross reactivity data shown in Table 1, estrone in the first wastewater sample would have contributed ~26, 8, and 12 ng L^{-1} of 17ß-estradiol signal to the A1, A2, and A3 assays, respectively. Likewise, estrone in the second set of wastewater samples would have contributed ~36, 11, and 16 ng L^{-1} to the 17ß-estradiol signal. If the estrone cross-

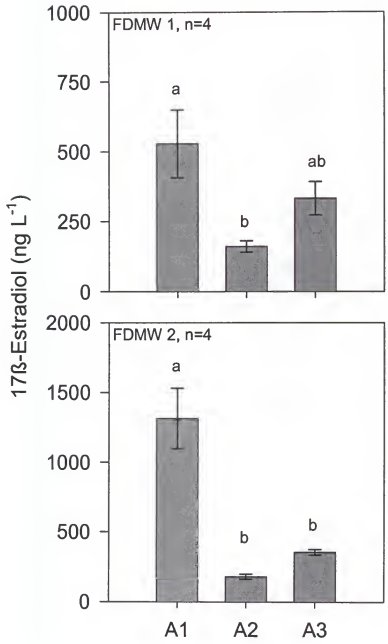


Figure 3-1. Apparent concentration of 17ß-estradiol in flushed dairy manure wastewater (FDMW) samples measured by three immunoassays. Different letters (a,b) indicate a significant difference ($\alpha = 0.05$) between sample means. Error bars denote standard error of the mean.

reactivity data provided by the manufacturers are correct and the EIA measured estrone concentrations are accurate, the large differences observed between assays do not appear to be caused by estrone cross-reactivity.

Other types of matrix interferences that are known to affect the quality of EIA data are often associated with coextracted humic substances. For example, Huang and Sedlak (*73*) demonstrated that certain types of humic substances extracted from surface water could give positive signals during 17ß-estradiol EIA. Presumably, the humic substances cross-react with the 17ß-estradiol antibody or adsorb to the estradiol enzyme conjugate in a manner that inhibits the competitive antibody binding and thus give a false-positive EIA signal. On the other hand, humic substances may cause false-negative EIA signals if they inhibit the competitive binding of 17ß-estradiol to the antibody binding sites.

Ideally, the lack of agreement between immunoassays could be reconciled with a more conclusive measurement technique like gas chromatography-mass spectrometry (GC-MS) to determine which assay provided the most accurate measurement of 17ß-estradiol in FDMW. Unfortunately, GC-MS quantification was not possible with these wastewater samples due to the extraordinary sample complexity associated with the ether extracts and because the ng L^{-1} sample concentrations are several orders of magnitude lower than the detection limits (~10 µg L^{-1}) associated with the only published method for the GC-MS analysis of dairy wastes (*6*). A similar problem was reported by Raman et al. (6) who tried to compare the endogenous concentration of 17ß-estradiol in press cake dairy solids measured by the A1 EIA and GC-MS. Endogenous 17ß-estradiol could not be measured by GC-MS due to the relatively poor detection limits. However, when 17ß-estradiol was spiked into the press-cake samples, the A1 EIA and GC-MS methods

agreed well. Nevertheless, the spiked EIA and GC-MS comparison does not yield much information regarding bias of the A1 assay because an interference, if present, would have been greatly masked by dilution of the spiked samples.

Conclusions

Ether extraction and quantitation by EIA is a convenient method for measuring estrogens in FDMW. Although no differences were observed between EIAs when a pure solution of 17ß-estradiol was analyzed, three EIAs gave different 17ß-estradiol results for the same wastewater samples. The differences are most likely caused by one or more matrix interferences associated with coextracted humic substances in the sample. The poor quality of the ether extracts and low concentrations of 17ß-estradiol in the wastewater prevented GC-MS quantitation and therefore it is not known which of the three EIAs yielded the most accurate measurement of 17ß-estradiol. Based on the large differences observed between EIAs in this study, caution should be observed when interpreting the biological significance or ecological risk of 17ß-estradiol concentrations in livestock wastes when measured by EIA. Immunoassays are potentially valuable tools for the rapid screening of environmental samples. However, a better understanding of the artifacts and interferences associated with highly complex and variable livestock waste matrices are clearly needed. Future research should develop better extraction and/or purification techniques so that 17ß-estradiol and other estrogens can be measured in FDMW by more conclusive techniques like GC-MS or liquid chromatography-mass spectrometry (LC-MS) and to ensure that immunoassay results are accurate.

CHAPTER 4
DETERMINATION OF STEROIDAL ESTROGENS IN FLUSHED DAIRY MANURE WASTEWATER BY GC-MS AND COMPARISON WITH IMMUNOASSAY

Introduction

Livestock manure contains appreciable amounts of natural steroidal estrogen hormones, such as estradiol, estrone, and estriol, that can potentially contaminate surface and groundwater (*1-8*). Estrogen contamination of water resources is a concern because low part per trillion concentrations (10 to 100 ng L^{-1}) of these chemicals can adversely affect the reproductive biology of aquatic vertebrates such as fish, turtles, and frogs, by disrupting the normal function of their endocrine systems (*9-13,139*).

The ecological hazards, if any, posed by steroidal estrogens resulting from dairy production is not clearly known. Nevertheless, based on the amount of estrogens excreted in urine and feces, Lange et al. (*158*) estimated that pregnant and cycling cows are responsible for about 90% of the steroidal estrogen input to the environment by domestic livestock in the United States and Europe. Therefore, it is critically important to know the types and amounts of steroidal estrogens that occur in dairy wastes so that any potential endocrine disruption risks can be minimized or avoided.

Gauging the steroidal estrogen profile of dairy manure or any type of livestock waste is not a trivial task, however, due to the low concentrations that must be measured, the difficulties associated with extracting estrogens from manure, the chemical complexity of the resulting extract matrix, and the potential for degradation losses to occur during sample storage (*6*). Fluorometric, immunoassay, and chromatographic

methods have been used for the quantification of estrogens in dairy wastes (*6,83,144,147*). Of these techniques, immunoassay is the most popular method of determination owing to the widespread commercial availability of estrogen immunoassay kits, ease of use, pg mL^{-1} detection limits, and a general lack of sensitive chromatographic quantitation methods. The advantages of EIA can be offset, however, if their accuracy and reliability is compromised by interferences resulting from cross reactivity, enzyme inhibition, matrix effects (pH, ionic strength, humic substances), endogenous enzymes, and chromagens (*73,112-115,211*). Interferences associated with the immunoassay analysis of 17ß-estradiol in environmental samples is largely uninvestigated, but Chapter 3 showed that the measured concentrations of 17ß-estradiol in flushed dairy manure (FDMW) differed according to the brand of enzyme immunoassay (EIA) used for quantitation. The differences appeared to be caused by matrix interference, but could not be resolved due to lack of a sensitive chromatographic procedure for comparison.

Few GC-MS or liquid chromatography-mass spectrometry (LC-MS) based methods have been proposed for measuring estrogens in livestock wastes (*6,103*). To my knowledge, only one GC-MS method has been published for quantifying estrogens in dairy wastes (*6*). The sample preparation involved liquid-liquid ether extraction of the dairy waste sample followed by BSTFA [N, O-bis(Trimethylsilyl)fluoroacetamide] derivatization and GC-MS analysis. Unfortunately, the detection limits (~10 μg L^{-1}) for estrogens associated with the method of Raman et al. (6) is poor relative to the endogenous concentrations of steroidal estrogens (ng L^{-1}) found in FDMW (Chapter 3).

To better understand the types and amounts of steroidal estrogens existing in FDMW and to reveal any potential limitations of EIA, a highly sensitive and reliable analytical procedure is needed. The objective of this study was to develop a method that allows measurement of 17α-estradiol, 17ß-estradiol, estrone, and estriol in FDMW by GC-MS. The concentrations of 17ß-estradiol measured by GC-MS were compared with 17ß-estradiol concentrations measured by two commercially-available EIAs.

Materials and Methods

Chemicals and Reagents

Estrone, 17α-estradiol, 17ß-estradiol, and estriol were purchased from Sigm-Aldrich (St. Louis, MO). Methanol (HPLC-grade), methylene chloride (HPLC-grade), acetone (Optima grade), water (HPLC-grade), and formic acid (ACS-grade) were purchased from Fisher Scientific (Pittsburgh, PA). Sample reservoirs (75 mL), filtration frits (~20 µm), 500 mg Carbograph (graphitized carbon) solid-phase extraction (SPE) columns, 1000 mg C18 (octadecylsiloxane-bonded silica) high-flow SPE columns, and nylon syringe filters (13 mm, 0.2 µm) were purchased from Alltech Associates (Deerfield, IL). Immediately prior to use, the Carbograph columns were conditioned sequentially with 10 mL methylene chloride:methanol (80:20 v:v), 5 mL methanol, and 10 mL of pH 2 water and the C18 columns were conditioned sequentially with 5 mL acetone and 5 mL water.

Enzyme immunoassay kits for the quantitative determination of 17ß-estradiol were purchased from Assay Design, Inc. (cat. no. 900-008; Ann Arbor, MI) and Diagnostics Systems Laboratories, Inc. (cat. no. DSL-10-4300; Webster, TX). The immunoassay kits were designated A1 and A2 respectively. Bulk assay buffer for sample reconstitution and preparation of the assay calibration curve was included with the A1 EIA kit. Bulk assay

buffer (DSL 7401) was purchased from Diagnostics Systems Laboratories, Inc. for sample reconstitution and preparation of the assay calibration curve.

Sample Collection

Many dairies use hydraulic flushing for manure management, followed by primary treatment (mechanical screening or sedimentation, or both) to remove coarse solids. The liquid fraction of flushed dairy manure after settleable solids are removed is referred to as FDMW (*203*). A 1 L grab sample of FDMW was collected on 5 consecutive days (01/19/04 to 01/23/04) from the University of Florida Dairy Research Unit located at Hague, FL and transported on ice in less than 1 h to the laboratory in Gainesville, FL and immediately extracted. The total solids content of the FDMW sample collected each day was determined by the methods of APHA (*204*). The total solids content of the FDMW samples collected from each day was 0.79, 0.74, 1.04, 0.66, 1.31, and 0.91%, respectively.

Liquid Extraction

Eight aliquots (40 mL each) of the bulk FDMW sample were subsampled into separate 50 mL Teflon tubes and centrifuged at 15,000 *g* for 15 min to pelletize suspended solids. The clarified supernatant was transferred into a 125 mL flask without disturbing the pellet and set aside. Estrogens adsorbed to pelletized solids were extracted with 10 mL methanol in a 40°C ultrasonic bath for 30 min. After centrifugation at 4,000 *g* for 15 min, the methanol extract was combined with the aqueous portion of the sample and set aside. The pellet was extracted once more with 10 mL of methanol for 30 min in a 40°C ultrasonic bath, and after centrifuging 4,000 *g* for 15 min, the methanol extract was added to the previous supernatant and mixed thoroughly.

Solid-phase extraction efficiency was measured each day by spiking four of the eight aqueous-methanol supernatants with 40 ng each of 17α-estradiol, 17ß-estradiol, estrone, and estriol from a 1000 ng mL^{-1} stock solution prepared in acetone. An additional set (n=4) of spikes (20, 40, 60, and 80 ng of 17α-estradiol, 17ß-estradiol, estrone, and estriol) was included with FDMW 5 to assess the extraction efficiency at different spiking levels. Spiking was done after centrifugation and methanol extraction to minimize microbial degradation of the target analytes. Extraction efficiency was calculated by dividing the measured concentration of estrogens in the spiked sample by the theoretically expected concentration in spiked samples and the result was multiplied by 100.

Solid-Phase Extraction

Estrogens were extracted from the nonspiked and spiked samples using Carbograph solid-phase extraction (*47,105,164,212,213*). The samples were poured into fritted reservoirs and passed through preconditioned Carbograph SPE columns. The samples were percolated at 5 to 10 mL min^{-1} through the columns with the aid of a vacuum. Once the sample passed through, the flasks were rinsed with 50 mL of water and the rinse was applied to the columns. After rinsing, the reservoir was removed and the Carbograph column was washed sequentially with 5 mL of 75% methanol acidified with 100 mmol L^{-1} formic acid and 5 mL of 75% methanol. The base/neutral fraction of retained organics that included the target estrogens was eluted with 2 mL methanol and 15 mL of 80:20 (v:v) methylene chloride:methanol into 50 mL flasks. The captured eluant was heated at 70°C under a gentle stream of N_2 until the methylene chloride evaporated. After cooling, 50 mL of water was added to the residual methanol and mixed by swirling.

Sample Purification

To improve sample purity, C18 SPE was performed. The aqueous-solvent sample mixtures resulting from Carbograph extraction were poured into reservoirs and percolated at 5 to 10 mL min^{-1} through preconditioned C18 columns with the aid of vacuum. After the samples passed through, the flasks were rinsed with 50 mL of water and the rinse was applied to the C18 column. When the rinse passed through, vacuum was applied to the columns for an additional ~15 min to remove excess water. A nylon syringe filter was attached to the bottom of each C18 column and estrogens were eluted with 4 mL of acetone into preweighed sample vials. The final sample volumes were adjusted by weighing to 4.0 mL acetone, capped tightly, and stored at –20°C prior to subsampling for EIA and GC-MS analysis.

For EIA analysis, two 100 µL aliquots (one for each EIA) of acetone were removed from the nonspiked FDMW sample vials and placed into separate 5 mL evaporation flasks. The remaining non-spiked sample (3.8 mL) was immediately capped and stored at -20°C until GC-MS analysis. The 100 µL acetone aliquots were evaporated to dryness at 70°C under N_2. The dried sample was immediately reconstituted in 1 mL of the appropriate EIA assay buffer. The reconstitued samples were individually sonicated for ~1 min. to enhance solubilization in the assay buffer. The samples were poured into 1.5 mL microcentrifuge tubes, capped tightly, and stored at -20 °C prior to EIA analysis.

Enzyme Immunoassay Description

The A1 and A2 immunoassays were selected because they have been used previously for the quantification of 17ß-estradiol in dairy wastes (Chapter 3) (*6*). Both immunoassays use rabbit polyclonal antibodies (RPA) and have less than 5% cross reactivity with other natural steroidal estrogens (Table 1). Each assay uses the

competitive binding principle, whereby 17ß-estradiol and a fixed amount of enzyme labeled-estradiol compete for RPA binding sites. However, the A2 assay uses RPAs that are directly coated onto the microplate wells, whereas the A1 microplate wells are coated with goat anti-rabbit IgG to capture the 17ß-estradiol-RPA complex. The alkaline phosphatase and streptavidin-horseradish peroxidase enzyme tracers used by A1 and A2 assays respectively, are commonly-used enzyme reagents for estrogen immunoassay (*159,205-207*).

Each assay was performed according to the manufacturer's instructions except that calibration standards for the A2 EIA were prepared in the substitute buffer (DSL 7401) instead of serum by diluting a known concentration of 17ß-estradiol to six concentrations. All standards and samples were assayed in duplicate and an average value was used to generate standard curves and interpolate unknown sample concentrations. Microplate washing was performed with an ELx50/8 strip washer (Bio-Tek Instruments, Inc., Winooski, VT) with the wash buffer reagents provided in each kit. The absorbance values of each well were measured using an FL 600 microplate reader (Bio-Tek Instruments, Inc.). A four-parameter logistic equation was used for all standard calibration curves (*208*).

Immunoassay performance characteristics including sensitivity, standardization, precision, and recovery of diluted and spiked samples were evaluated on both days of wastewater analysis. Sensitivity is defined as the lowest measurable concentration of 17ß-estradiol that can be distinguished from the respective 0 pg mL^{-1} calibrator (95% confidence interval) associated with each EIA (*209*). Sensitivity was calculated for each

EIA by interpolation of the mean of eight replicate samples of the respective 0 pg mL^{-1} calibrator minus two standard deviatons.

Standardization accuracy refers to the ability of each EIA to yield a correct measurement of 17ß-estradiol for a known standard concentration. Standardization accuracy (external recovery %) was measured by preparing 2500, 1250, 625, and 312 pg mL^{-1} concentrations of 17ß-estradiol in the appropriate buffer solution for each assay. Four values were selected to ensure accurate recovery at different interpolation points along the standard curve. Recovery percentage at each concentration was calculated by dividing the measured sample concentration by the known sample concentration and multiplying the result by 100. The four resulting values were averaged to express the average percent recovery.

Intra-assay precision refers to the within-run reproducibility of the 17ß-estradiol signal that is produced for a particular sample in an EIA. Precision was evaluated by calculating the percent coefficient of variation (CV%) observed between duplicate measurements corresponding to the four neat wastewater samples from each day. The twenty resulting CV% values were averaged to express precision.

Recovery of diluted samples is a gauge of the linear relationship between 17ß-estradiol measured in diluted samples relative to the neat samples. Dilution recovery was evaluated by diluting one of the neat sample concentrations from each of the five FDMW with an equal volume of the appropriate buffer solution for each assay. Dilution recovery was expressed as a percentage by dividing the measured concentration of the diluted sample by the theoretically expected concentration of the diluted sample, and the result was multiplied by 100.

Table 4-1. Description and cross reactivity of two enzyme immunoassay systems used for measuring 17ß-estradiol in flushed dairy manure wastewater extracts.

Description	A1	A2
Assay principle	Competitive	Competitive
17ß-Estradiol antibody	rabbit polyclonal	rabbit polyclonal
Matrix	TBS†	DSL 7401
Conjugate/Enzyme	E2-ALP	E2-Biotin/SHRP
Substrate	p-NPP	TMB
Range (pg mL^{-1})	0-30,000	0-6,000
MDL (pg mL^{-1})	29	7
Precision (CV%)	9	4
Cross reactivity (%)		
17ß-Estradiol	100	100
17α-Estradiol	0.1	0.3
Estrone	4.6	1.4
Estriol	0.5	1.1

†TBS, Tris-buffered saline containing proteins and detergents and sodium azide as a preservative; E2, 17ß-estradiol; ALP, alkaline phosphatase; SHRP, streptavidin horseradish peroxidase; p-NPP, p-nitrophenol phosphate; TMB, tetramethylbenzidine; MDL, minimum detection limit.

GC-MS Analysis

The GC-MS analysis of estrone, 17α-estradiol, 17ß-estradiol, and estriol was performed by the University of Florida Analytical Toxicology Core Laboratory (ATCL). At the ATCL, an additional purification of the samples was performed using C18 SPE and the target estrogens were derivatized overnight with BSTFA in dimethylformamide for GC-MS analysis. The derivatized product was taken to dryness under N_2, reconstituted in 500 µL of acetonitrile, spiked with 10 µL of pyrelene (100 ng μL^{-1}; internal standard) and transferred to an amber vial for GC-MS (electron-impact ionization; positive ions). Analyte quantitation was performed in single ion monitoring mode (SIM) and was conducted against a five-point standard curve (1 to 500 ng) with a correlation coefficient ≥0.995. The ions selected for quantitation of the trimethylsilyl derivatives were *m/z* 416 for 17α-estradiol and 17ß-estradiol, *m/z* 342 for estrone, and *m/z*

504 for estriol. A full scan chromatogram of a FDMW sample is provided in Appendix A.

Data Analysis

The experimental design comparing GC-MS and EIA was a two-way factorial consisting of three analytical methods X five sampling times with four sample replications. Experimental data were analyzed using the General Linear Model program of SAS with a separation of sample means by Duncan's multiple range test (*210*).

Results and Discussion

Extraction Method Performance

Spiked recovery of 40 ng estrone, 17α-estradiol, 17ß-estradiol, and estriol averaged 101, 96, 125, and 99%, respectively (Table 2). As shown in Figure 1, the net amount of each estrogen extracted from FDMW after spiking with 20, 40, 60, and 80 ng was linear within the range evaluated. The method precision (RSD ≤12%) was also very good for all the target analytes (Table 2). Overall, the spiked recovery experiment demonstrates that Carbograph SPE and C18 purification is a reliable sample preparation method for the sensitive determination of estrogens in FDMW by GC-MS.

Table 4-2. Average recovery of spiked estrogens from five samples of FDMW.

FDMW	Estrone	17α-estradiol	17ß-estradiol	Estriol
	----------recovery†, % (RSD)----------			
1	92 (5)	96 (6)	116 (5)	90 (9)
2	104 (5)	105 (5)	134 (8)	99 (9)
3	105 (2)	93 (5)	121 (2)	109 (5)
4	107 (7)	94 (10)	139 (8)	107 (10)
5	98 (7)	94 (9)	114 (8)	90 (12)
avg.	101 (5)	96 (7)	125 (6)	99 (9)

FDMW, flushed dairy manure wastewater; †Mean values from four replicate samples.

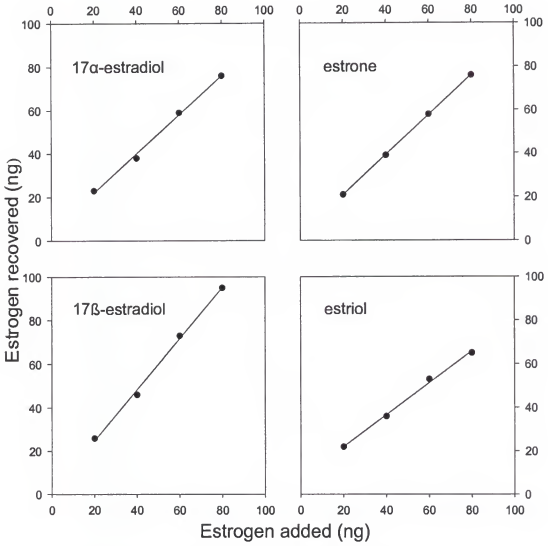


Figure 4-1. Net amount of estrone, 17α-estradiol, 17ß-estradiol, and estriol extracted from FDMW after spiking with 20, 40, 60, and 80 ng of target analytes.

The Carbograph-C18 extraction and purification method used in this study compares favorably with other research involving SPE of estrogens from environmental matrices. For example, Baronti et al.(*47*) reported ≥86% recovery of added 17ß-estradiol, estrone, and estriol from sewage influent, sewage effluent, and river water when using Carbograph SPE. Lee and Peart (*106*) reported ≥98% recovery of added 17ß-estradiol, estrone, and estriol from sewage effluent by C18 SPE.

GC-MS Analysis

The endogenous concentration of estrogens in five samples of FDMW determined by GC-MS is shown in Table 3. Estrone, 17α-estradiol, and 17ß-estradiol concentrations averaged 879, 2282, and 643 ng L^{-1}, respectively, but estriol was not detected during five consecutive sampling days. The absence of estriol and large abundance of 17α-estradiol relative to 17ß-estradiol and estrone is consistent with the estrogen excretion profile of cattle (*Bos Taurus*) (*144,146*).

Table 4-3. Estrogen concentrations in five samples of FDMW measured by GC-MS (n=4).

FDMW	Estrone	17α-Estradiol	17ß-estradiol	Estriol
	ng L^{-1} ± SE			
1	2356 ± 74	2036 ± 92	711 ± 52	BDL
2	467 ± 66	1750 ± 62	525 ± 42	BDL
3	650 ± 22	3270 ± 99	957 ± 22	BDL
4	370 ± 46	2114 ± 98	351 ± 17	BDL
5	551 ± 50	2239 ± 160	672 ± 32	BDL

FDMW, flushed dairy manure wastewater; SE, standard error of the mean; BDL, below detectable limits.

It is difficult to compare in a meaningful way the estrogen concentrations in FDMW with other types of dairy waste samples because FDMW is highly dilute. However, compared with other low solids content dairy wastes such as from holding

ponds, estrogen concentrations in FDMW appear to be less (*161*). For example, Williams (161) reported GC-MS measured concentrations of estrone, 17α-estradiol and 17ß-estradiol in dairy holding ponds averaged 7595, 5185, and 3350 ng L^{-1}, respectively. As mentioned previously, however, the detection limits associated with the sample preparation frequently hindered GC-MS quantification of estrogens and resulted in a high frequency of "below detectable limits" reported in several dairy waste samples (*6,161*). For example, 87 and 60% of samples collected from dairy holding ponds were below the method detection limits for 17ß-estradiol and estrone, respectively (*161*).

Immunoassay Performance

A summary of performance characteristics associated with the EIA analysis is shown in Table 4. Sensitivity was similar between EIAs, but somewhat greater than the estimated 29 pg mL^{-1} and 7 pg mL^{-1} data provided by the A1 and A2 manufacturers, respectively. No differences were observed between assays regarding the concentration of a known standard solution. The concentration of the stock solution was also verified by GC-MS. Therefore, the EIAs appeared to be well standardized with each other and to the GC-MS. The A2 assay was generally more precise than the A1 assay as evidenced by a lower CV% between replicate sample measurements. Dilution recovery demonstrated

Table 4-4. A summary of performance data resulting from the analysis of flushed dairy manure wastewater samples by two immunoassays.

Performance characteristic	A1	A2
Sensitivity (pg mL^{-1})	53	51
Standardization accuracy, n=4 (%)	95	95
Precision of replicate samples, n=20 (CV%)	26	7
Recovery of diluted samples, n=5 (%)	85	125

n= number of samples; NA= not applicable; CV= coefficient of variation.

reasonably accurate recovery of 17ß-estradiol at two different interpolation points evaluated from the standard curve. Overall, the performance data suggest that each assay was accurately calibrated and worked properly.

Immunoassay and GC-MS Method Comparison

The measured concentration of 17ß-estradiol in FDMW samples differed according to the analytical method used and day of sample collection (Figure 2). Because no differences were observed between the GC-MS and EIAs, or between EIAs when a pure solution of 17ß-estradiol was analyzed, it seems probable that humic substances coextracted with the estrogens from the FDMW by Carbograph-C18 SPE interfered with the EIA measurement of 17ß-estradiol by exerting a variable matrix effect. The humic substances appeared to cause imprecision in the A1 assay and a general false-negative bias in the A2 assay.

Other researchers have reported interference during 17ß-estradiol EIA associated with humic substances. Huang and Sedlak (73) reported that certain types of humic substances extracted from surface water interfered with 17ß-estradiol EIA. They demonstrated humic substances crossreacted with the 17ß-estradiol antibody and caused a false-positive 17ß-estradiol signal. It should be noted, however, that Huang and Sedlak (73) tested the crossreaction in the absence of 17ß-estradiol. Therefore, any bias that might occur during the EIA analysis of surface water samples was not clearly established. It can be speculated that coextracted humic substances in the sample might adsorb to the 17ß-estradiol in the sample solutions, thereby reducing the availability of 17ß-estradiol for binding to the anti-estradiol antibody and causing a false-negative EIA response. Because the Carbograph-C18 SPE procedure extracts hydrophobic molecules, including

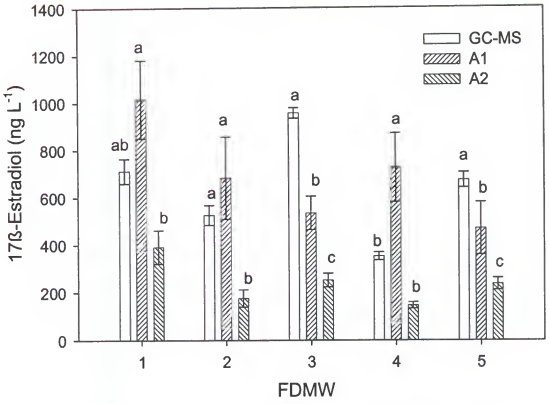


Figure 4-2. Apparent concentration of 17ß-estradiol in five flushed dairy manure wastewater (FDMW) samples measured by GC-MS and two enzyme immunoassays. The letters (a,b,c) indicate a significance difference ($\alpha = 0.05$) between analytical methods for a particular FDMW sample. Error bars denote standard error of the mean.

17ß-estradiol (log K_{ow}=3.1 to 4.0) (*29-31*), it seems reasonable that hydrophobic interactions between 17ß-estradiol and the coextracted humic substances might occur.

Based on the differences observed between EIAs and between EIAs and GC-MS in this study, caution should be used when interpreting the biological significance or ecological risk of 17ß-estradiol concentrations measured by EIA. Immunoassays are potentially valuable tools for the rapid screening of environmental samples. However, a better understanding of the artifacts and interferences associated with highly complex and variable matrices associated with livestock wastes is clearly needed.

Conclusions

A new sample preparation method involving liquid and solid-phase extraction was developed for the measurement of estrone, 17α-estradiol, 17ß-estradiol, and estriol in FDMW by GC-MS. Recovery of each estrogen was >90% as determined by spiking experiments. Characterization of the estrogen profile of FDMW revealed a large abundance of 17α-estradiol relative to 17ß-estradiol and estrone. Estriol was not detected in FDMW. The concentration of 17ß-estradiol measured in FDMW by GC-MS was compared with measurements from two EIAs. The EIA and GC-MS data agreed poorly. The unreliable 17ß-estradiol concentrations reported by EIA appeared to be caused by matrix interference. Future research involving quantitative EIA should use a GC-MS or LC-MS validation program to ensure that immunoassay data are accurate.

CHAPTER 5
PRELIMINARY DETERMINATION OF STEROIDAL ESTROGENS IN SURFACE AND GROUNDWATER AT A DAIRY BY GC-MS

Introduction

Livestock wastes are increasingly recognized as a source of endocrine disrupting compounds such as natural steroidal estrogens (e.g., estrone, 17α-estradiol, 17ß-estradiol, and estriol) to surface and groundwater resources (*1-5,7,8*). Estrogens are an environmental concern because low part per trillion concentrations (10 to 100 ng L^{-1}) in water can adversely affect the reproductive biology of aquatic vertebrate species including fish, frogs, and turtles (*9-13,139*). A number of studies have demonstrated the sensitivity of fish to estrogen exposure. For example, Metcalfe et al. (139) reported that Japanese medaka (*Oryzias latipes*) fish developed intersex (testis-ova) or suffered complete sex reversal when exposed to either 17ß-estradiol (~10 ng L^{-1}) or estrone (~10 ng L^{-1}) in the laboratory. Panter et al. (*11,12*) reported that 17ß-estradiol concentrations in water ≥30 ng L^{-1} for 21 d can induce vitellogenin synthesis and abnormal testicular growth in male fathead minnows (*Pimephales promelas*).

At present, little is known about the potential harm, if any, to fish and wildlife caused by estrogens originating from livestock wastes. However, Irwin et al. (*13*) reported that vitellogenin production by female painted turtles (*Chrysemys picta*) in ponds was significantly affected by estrogens in beef cattle runoff compared with turtles in ponds unexposed to beef cattle runoff. Although biological studies of estrogen contamination of water resources by livestock wastes have not been widely investigated,

some researchers have reported alarming concentrations of 17ß-estradiol in manure-impacted surface and groundwater. For example, Nichols et al. (*4*) reported 17ß-estradiol concentrations ≤1280 ng L^{-1} in runoff from a poultry litter amended soil. Peterson et al. (*201*) reported 17ß-estradiol concentrations ≤66 ng L^{-1} in five springs of northwest Arkansas (a major poultry and cattle production region).

The potential contamination of water resources by steroidal estrogens originating from livestock production facilities is an issue that warrants careful attention. However, the accurate measurement of steroidal estrogens in environmental samples is a diffult task due to the low ng L^{-1} concentrations that must be measured and the chemical complexity of samples resulting from the extraction of surface and groundwater (*6,47,103*). A number of researchers have used enzyme-immunoassay (EIA) techniques to measure the occurrence of 17ß-estradiol in manure-impacted waters (*3,4,201,214*). However, previous work showed that EIA results can be inaccurate due to coextracted matrix interferences (Chapter 3, Chapter 4). Even if EIA's can be validated, they are usually specific for a single analyte such as 17ß-estradiol. This is a limitation because other steroidal estrogens such as estrone, 17α-estradiol, and estriol may also affect water quality. Therefore, more conclusive measurement techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) are preferable to EIA. In Chapter 4, a new method was developed for the measurement of estrogens in flushed dairy manure wastewater. The objective of this study was to determine if the procedure could be adapted for the analysis of surface and groundwater, so a preliminary method was developed and a survey experiment was performed.

Materials and Methods

Chemicals and Reagents

Estrone, 17α-estradiol, 17ß-estradiol, and estriol were purchased from Sigm-Aldrich (St. Louis, MO). Methanol (HPLC-grade), methylene chloride (HPLC-grade), acetone (Optima grade), water (HPLC-grade), and formic acid (ACS-grade) were purchased from Fisher Scientific (Pittsburgh, PA). Sample reservoirs (75 mL), filtration frits (~20 μm), 500 mg Carbograph (graphitized carbon) solid-phase extraction (SPE) columns, 1000 mg C18 (octadecylsiloxane-bonded silica) high-flow SPE columns, and nylon syringe filters (13 mm, 0.2 μm) were purchased from Alltech Associates (Deerfield, IL). Immediately prior to use, the Carbograph columns were conditioned sequentially with 10 mL methylene chloride:methanol (80:20 v:v), 5 mL methanol, and 10 mmol L^{-1} HCl acidified water pH 2) and the C18 columns were conditioned sequentially with 5 mL acetone and 5 mL water.

Sample Collection

Surface water and groundwater were collected from the University of Florida Dairy Research Unit (DRU) located near Hague, FL. Sampling coordinates and chemical characteristics of the water samples are provided in Appendix B. Bulk grab samples (4 L) of surface water were collected on 1/29/04 from four locations designated SW1, SW2, SW3, and SW4, respectively. Of the four sampling locations, only SW4 was directly impacted by a small herd (~25) of grazing cattle that was not fenced from the stream. The SW3 sampling location was less than 10 m from the pit that collected flushed dairy manure wastewater. The SW2 location was ~1 km downstream from SW 3 and was collected at the intersection of county road 237. The SW1 location was associated with row crops and was also collected at the intersection of county road 237.

Bulk grab samples (4 L) of groundwater were collected on 2/2/04 from four wells less than 6 m deep that were designated GW1, GW2, GW3 and GW4, respectively. Of the four groundwater sampling locations, the most likely to be directly contaminated was GW4 because it was near the confinement facility and the flushed dairy manure wastewater holding pit. The GW1 well should represent background groundwater concentrations since it was near a wooded area that did not support cattle grazing or receive land-application of manure. The GW2 well was associated with a sprayfield that received regular applications of FDMW. The GW3 well was associated with fallow land that did not receive applications of dairy waste. After collection, all water samples were transported on ice in less than 2 h to the laboratory in Gainesville, FL and extracted immediately.

Filtration and Spiking

Bulk water samples were passed through a 20 μm filter to remove suspended particulate matter. Each filtered sample was subsampled (200 mL) four times into separate 250 mL flasks. To measure extraction efficiency, four additional 200 mL aliquots of SW1 and GW1 were collected from the bulk filtered samples and spiked with 40 ng each of estrone, 17α-estradiol, 17ß-estradiol, and estriol. Spiking was done after filtration to minimize microbial degradation of the target analytes. Extraction efficiency was calculated by dividing the measured estrogen concentration of the spiked sample by the theoretically expected concentration in spiked samples and the result was multiplied by 100.

To assess the potential GC-MS signal interference, four additional 200 mL aliquots of SW1 and GW1 were collected from the bulk filtered samples and processed simultaneously. Extraction efficiency and the potential for GC-MS interference was also

evaluated using HPLC water that was processed in the same manner as the surface and groundwater samples.

Extraction

Estrogens were extracted from all water samples using Carbograph SPE columns (*47,105,164,212,213*). The samples were percolated at a rate of 10 to 20 mL min^{-1} through the Carbograph with the aid of vacuum. Once the sample passed through, the flasks were rinsed with 50 mL of water and the rinse was applied to the columns. After the rinsing, the Carbograph column was washed sequentially with 5 mL of 75% methanol acidified with 100 mmol L^{-1} formic acid and 5 mL of 75% methanol. The base/neutral fraction of retained organics that included the target estrogens was eluted with 2 mL methanol and 15 mL of 80:20 (v:v) methylene chloride:methanol into 50 mL flasks. The captured eluant was heated at 70°C under a gentle stream of N_2 until the methylene chloride evaporated. After cooling, 50 mL of water was added to the residual methanol and mixed by swirling.

Sample Purification

To improve sample purity, C18 SPE was performed. The aqueous-solvent sample mixtures resulting from Carbograph extraction were percolated at a rate of 5 to 10 mL min^{-1} through preconditioned C18 columns with the aid of vacuum. After the samples passed through, the flasks were rinsed with 50 mL of water and the rinse was applied to the C18 column. When the rinse passed through, vacuum was applied to the columns for about 15 min to remove excess water. A nylon syringe filter was attached to the bottom of each C18 column and estrogens were eluted with 4 mL of acetone into preweighed sample vials. The final sample volumes were adjusted by weighing to 4.0 mL acetone, capped tightly, and stored at –20°C prior to GC-MS analysis.

GC-MS Analysis

The GC-MS analysis of estrone, 17α-estradiol, 17ß-estradiol, and estriol was performed by the University of Florida Analytical Toxicology Core Laboratory (ATCL). At the ATCL, an additional purification of the samples was performed using C18 SPE and the target estrogens were derivatized overnight with BSTFA in dimethylformamide for GC-MS analysis. The derivatized product was taken to dryness under N_2, reconstituted in 500 μL of acetonitrile, spiked with 10 μL of pyrelene (100 ng μL^{-1}; internal standard) and transferred to an amber vial for GC-MS (electron-impact ionization; positive ions). The four samples of SW1 and four samples of GW1 that were designated for evaluating GC-MS interference were spiked with 40 ng each of estrone, 17α-estradiol, 17ß-estradiol, and estriol. Interference of the GC-MS signal at the particular spiking concentration was expressed as a recovery percentage by dividing the measured estrogen concentration of the spiked sample by the theoretically expected concentration in spiked samples and the result was multiplied by 100. Analyte quantitation was performed in single ion monitoring mode (SIM) and was conducted against a five-point standard curve (1 to 500 ng) with a correlation coefficient ≥0.995. The ions selected for quantitation of the trimethylsilyl derivatives were *m/z* 416 for 17α-estradiol and 17ß-estradiol, *m/z* 342 for estrone, and *m/z* 504 for estriol. A full scan chromatogram of a surface and groundwater sample is provided in Appendix A.

Results and Discussion

Interference

A positive interference of the GC-MS signal was observed at the 40 ng spiking level for each of the target estrogens in all types of water samples evaluated (Table 1). The interference was particularly significant for estrone, ranging from 180 to 287%. The

cause of the observed interference is unknown. However, considering that the problem was noted in HPLC water as well as the surface and groundwater samples, it seems likely that the problem was more of an instrumentation issue rather than a problem with sample purity. It cannot be ruled out, however, that trace amounts of substances in the HPLC water, solvents, or possibly from the glassware or SPE columns caused or contributed to the signal interference. More work is needed to resolve the source of the interference and to take steps towards eliminating the problem or make use of suitable calibration samples.

Table 5-1. Interference observed with the GC-MS analysis of spiked water samples.

	Surface water		Groundwater		HPLC water	
Analyte	†recovery %	RSD	recovery %	RSD	recovery %	RSD
Estrone	287	6	180	18	201	27
17α-estradiol	150	20	161	14	158	19
17ß-estradiol	137	15	140	14	127	11
Estriol	146	14	151	18	145	23

†Mean values from four replicate samples; RSD, relative standard deviation.

Extraction Method Performance

In light of the interference observed for each of the signals associated with the target analytes, the extraction efficiency has to be evaluated in a manner that takes into account the contribution of the interference. Thus, to estimate extraction efficiency, the recovery calculations were adjusted downward in proportion to the interference observed for each target analyte in each matrix. The estimated recovery of estrogens spiked into water samples was ≥77% for each target analyte (Table 2). Method precision was also very good; RSD was ≤16% for all the target analytes. The spiked recovery experiment demonstrated that Carbograph SPE and C18 purification is likely an efficient sample preparation method for the GC-MS analysis of steroidal estrogens in surface and

Table 5-2. Estimated recovery of estrogens added to water samples.

	Surface water		Groundwater		HPLC water	
Analyte	†recovery %	RSD	recovery %	RSD	recovery %	RSD
Estrone	99	9	88	7	92	5
17α-estradiol	100	10	90	12	94	8
17ß-estradiol	99	7	87	9	91	5
Estriol	98	9	77	16	85	8

†Mean values from four replicate samples; RSD, relative standard deviation.

groundwater, but that more work needs to be done to resolve interference so that the method can be validated. The extraction efficiency reported here compares favorably with a number of reports involving SPE of estrogens from natural waters. For example, Lagana et al (*105*) reported ≥82% recovery of added 17ß-estradiol, estrone, and estriol from 1 L of both groundwater and river water.

Survey of Surface and Groundwater

Except for the surface water sample collected from the highly impacted site (SW4) and the groundwater sample collected from the non-impacted location (GW1), estrogens were either not detected or were below the limits of quantitation in the water samples. Estrone measured 60 ng L^{-1} in both the SW4 and GW1 samples. However, considering the significant amount of interference that was observed with estrone, it seems likely that the measured concentrations of estrone are inaccurate. If estrone was present in the samples from the impacted site, concentrations were not larger than the concentrations measured in the nonimpacted groundwater. Clearly, the survey of surface and groundwater at the dairy suggests that manure-borne estrogens were not grossly affecting the water quality at the time of sampling. This suggestion appeared true even for locations where cows directly impacted the surface water. Refinement of the current method and a more extensive survey of the waters at the dairy is needed to provide

definitive proof of any estrogen contamination at the site investigated. However, the result that no measurable estrogen concentrations were found in the surface or groundwater water is consistent with previous research that has demonstrated rapid dissipation of estrogens in soil, sediment, and water due to biodegradation and sorption (*64,168*).

Few studies have measured estrogen concentrations in manure-impacted waters by GC-MS for comparison with the current results. However, Fine et al. (*103*) measured estrogens in groundwater monitoring wells at a few swine farms. They detected a measurable amount of estrone (4.5 ng L^{-1}) in only one groundwater sample that was collected from a shallow well adjacent to a stock tank for watering cattle. The authors did not clearly indicate if the contamination was due to leakage from swine lagoons or from cattle excretion, but nevertheless, a small concentration of estrone was detected in the groundwater. Kolpin et al. (*200*) reported estrogen concentrations $\leq$200 ng L^{-1} in a network of 139 streams in 30 states impacted by urban and livestock wastes. In general, however, estrogens occurred infrequently in the majority of the samples tested. For example, estrone, 17α-estradiol, 17ß-estradiol, and estriol concentrations were reported in only 7, 6, 10, and 21% of 70 stream water samples measured.

Conclusions

A method development and survey experiment was conducted for the purpose of measuring estrogens in surface and groundwater by GC-MS. During method development, it was found that interference affected GC-MS quantification of estrogens in surface and groundwater. However, the sample preparation method used appeared promising because, after accounting for interference, excellent extraction efficiencies ($\geq$77%) with low RSD (16%) were observed. A survey of surface and groundwater at a

dairy farm for estrogens revealed that estrone may have been present in stream water that was directly impacted by cattle, but that estrone concentrations did not exceed the concentration of estrone detected in a sample of groundwater from a non-impacted location. Measurable amounts of 17α-estradiol, 17ß-estradiol, or estriol were not found in any of the water samples tested. Therefore, estrogens of livestock origin do not appear to be grossly affecting the water quality at the dairy farm studied. Further refinement and validation of the method is needed for more conclusive studies of estrogens in manure-impacted surface and groundwater.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The accurate measurement of steroidal estrogens in environmental matrices such as flushed dairy manure wastewater (FDMW), surface water, and groundwater is a difficult task. Liquid extraction of 17ß-estradiol from FDMW with ether and analysis by three different enzyme immunoassays revealed that matrix interference significantly affected the accuracy of one or all of the assays. The complexity of the ether extracts prevented comparison of the immunoassay data with gas chromatography-mass spectrometry (GC-MS). Based on the results, a more extensive sample preparation method involving chromatographic purification was deemed necessary so that estrogens could be measured by GC-MS.

A new method based on liquid and solid-phase extraction was developed that enabled ng L^{-1} measurements of four endogenous estrogen hormones (estrone, 17α-estradiol, 17ß-estradiol, and estriol) in FDMW by GC-MS. Three estrogens were present at measurable concentrations in FDMW including estrone, 17α-estradiol, and 17ß-estradiol. The GC-MS measured concentrations were compared with the results of two immunoassays. Neither immunoassay provided data that consistently agreed with GC-MS. The poor agreement was attributed to matrix interference that appeared to be associated with coextracted humic substances.

To address concerns regarding the possible estrogen contamination of surface and ground water at a dairy, the new method was adapted for water samples and a survey experiment was conducted. During method development, it was found that interference

affected GC-MS quantification of estrogens in water samples. However, the sample preparation method appeared promising because, after accounting for interference, excellent extraction recoveries were observed. Measurable concentrations of 17α-estradiol, 17ß-estradiol, or estriol were not found in surface or groundwater at the dairy. Some estrone was detected in stream water that was directly impacted by cows. However, a similar concentration of estrone was also measured in groundwater from a non-impacted location. Further refinement and validation of the method is needed for more conclusive studies of estrogens in manure-impacted water.

In conclusion, this study addressed three areas of critical research needs: 1) the development and validation of a sensitive and flexible method for measuring estrogens in dairy wastes by GC-MS, 2) the characterization of the estrogen profile of a particular type of dairy waste (e.g., FDMW), and 3) method development for the analysis of estrogens in dairy waste-impacted surface and groundwater. Future research should work towards standardization of sample preparation and analytical methods for measuring estrogens in environmental matrices. If immunoassays are to be used for measuring estrogens in environmental samples, then more work needs to be done to resolve interferences from humic substances to ensure that the results are valid. Future research should also include the measurement of the glucuronide and sulfate conjugates of estrogens. The sample preparation method developed in this study should be adaptable to conjugated estrogens, except that a hydrolysis procedure is required prior to GC-MS analysis. Many types of dairy wastes (e.g., separated solids, holding ponds, anaerobically-digested FDMW) need to be characterized so that estrogen concentrations associated with manure handling and storage practices can be evaluated. The sample

preparation method used in this study should be adaptable to the analysis of other dairy waste samples. More extensive surveys of impacted and nonimpacted surface and groundwater resources are needed to determine if manure-borne estrogens affect water quality or adversely affect exposed organisms. The incorporation of bioassay methods in water quality surveys and/or studies of fish and wildlife collected from manure-impacted sites may help determine if estrogen contamination of waterways is a biological or ecological concern. Future experiments should be designed to evaluate the degradation and sorption of estrogens in manure, soil, and water. Again, the methods developed in this study should provide a solid foundation for these future research endeavors.

APPENDIX A
GC-MS CHROMATOGRAMS

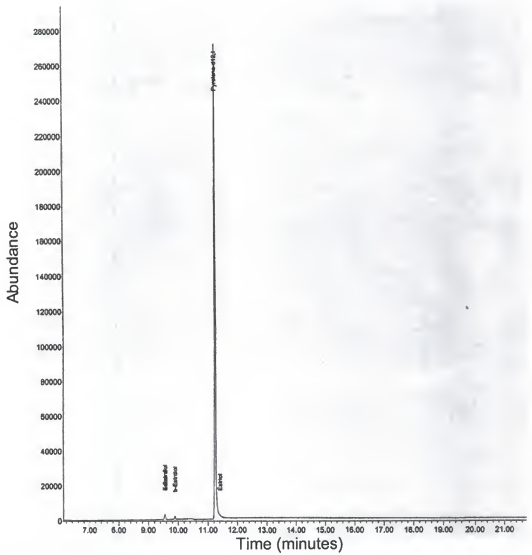


Figure A-1. GC-MS (full scan) chromatogram of the 25 ng calibration standard.

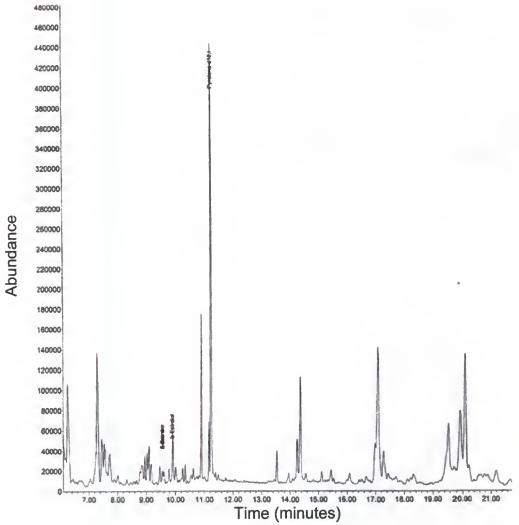


Figure A-2. GC-MS (full scan) chromatogram of a non-spiked flushed dairy manure wastewater sample.

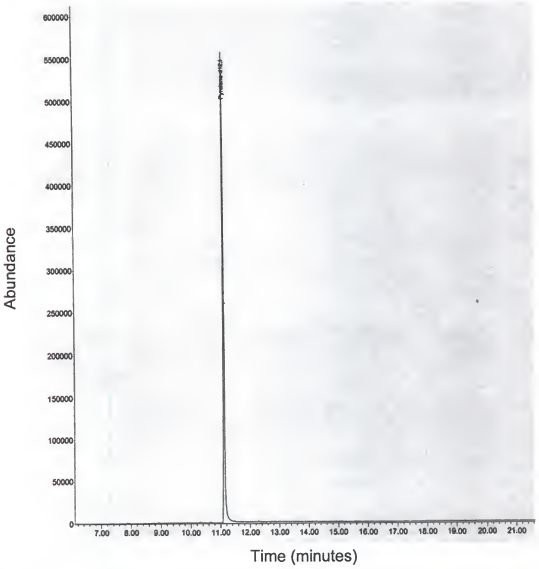


Figure A-3. GC-MS (full scan) chromatogram of a non-spiked HPLC water sample.

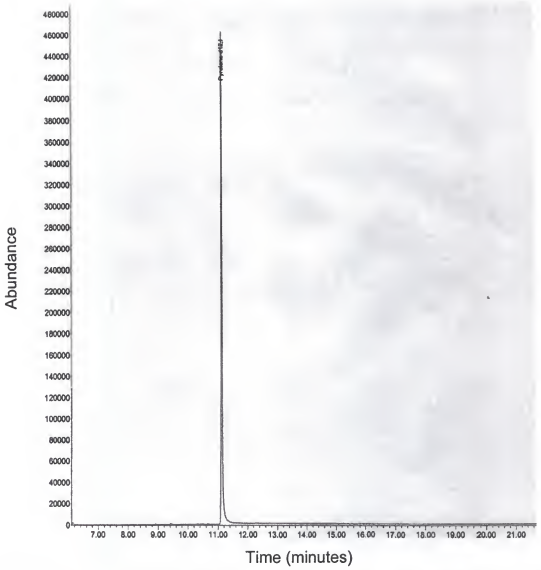


Figure A-4. GC-MS (full scan) chromatogram of a non-spiked groundwater (GW1) sample.

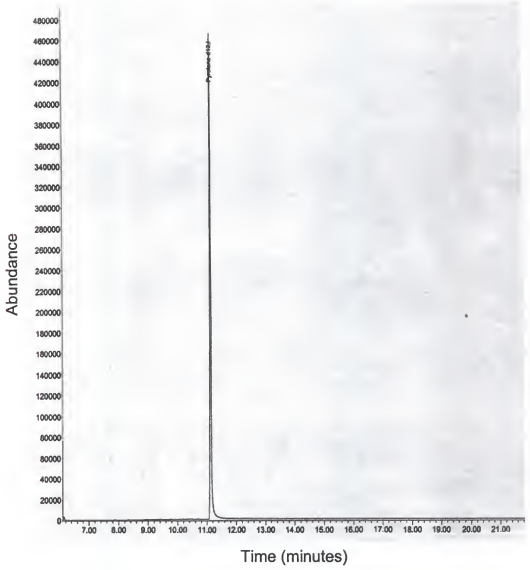


Figure A-5. GC-MS (full scan) chromatogram of a non-spiked surface water (SW1) sample.

APPENDIX B
SAMPLING LOCATIONS AND WATER CHARACTERISTICS

Table B-1. Coordinates of the surface and groundwater sampling locations.

	Latitude	Longitude
SW1	N 29° 46.505′	W 82° 25.294′
SW2	N 29° 46.669′	W 82° 25.298′
SW3	N 29° 46.816′	W 82° 24.959′
SW4	N 29° 48.014′	W 82° 24.939′
GW1	N 29° 46.253′	W 82° 24.668′
GW2	N 29° 47.395′	W 82° 25.223′
GW3	N 29° 47.321′	W 82° 25.588′
GW4	N 29° 46.845′	W 82° 24.932′

SW, surface water; GW, groundwater.

Table B-2. Selected chemical characteristics of surface and groundwater sampled at the University of Florida Dairy Research Unit.

	pH	EC	TOC	PO_4-P	NH_4-N	NO_3-N
		$\mu S\ cm^{-1}$	----------	$mg\ L^{-1}$	----------	
SW1	8.02	522	31.18	4.77	8.44	0.07
SW2	8.50	335	13.83	0.30	0.04	4.08
SW3	8.32	370	15.36	0.47	3.58	1.28
SW4	7.60	394	48.72	1.13	39.41	<0.03
GW1	7.34	195	5.82	<0.03	0.05	<0.03
GW2	7.17	356	3.47	<0.03	0.08	1.61
GW3	7.21	160	2.79	0.39	<0.03	4.53
GW4	8.06	257	2.39	<0.03	0.08	0.24

EC, electrical conductivity; TOC, total organic carbon; SW, surface water; GW, groundwater.

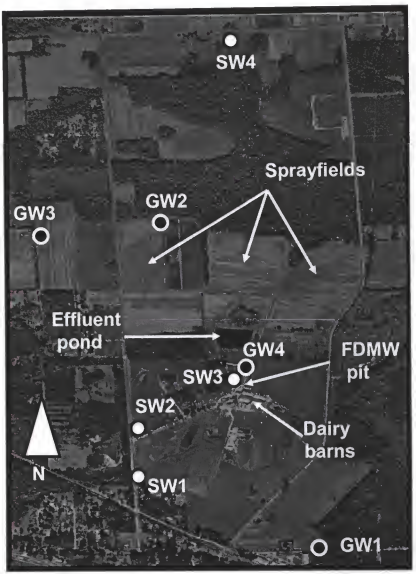


Figure B-1. Map of surface water (SW○) and groundwater (GW●) sampling locations at the University of Florida Dairy Research Unit.

LIST OF REFERENCES

1. Bushee, E. L., D. R. Edwards, and P. A. Moore. 1998. Quality of runoff from plots treated with municipal sludge and horse bedding. Trans. ASAE. 41:1035-1041.

2. Dyer, A. R., D.R. Raman, M.D. Mullen, R.T. Burns, L.B. Moody, A.C. Layton, and G.S. Sayler. 2001. Determination of 17ß-estradiol concentrations in runoff from plots receiving dairy manure. ASAE Meeting Paper No. 01-2107. St. Joseph, Mich.:ASAE.

3. Finlay-Moore, O., P. G. Hartel, and M. L. Cabrera. 2000. 17ß-Estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. 29:1604-1611.

4. Nichols, D. J., T. C. Daniel, P. A. Moore, D. R. Edwards, and D. H. Pote. 1997. Runoff of estrogen hormone 17ß-estradiol from poultry litter applied to pasture. J. Environ. Qual. 26:1002-1006.

5. Nichols, D. J., T. C. Daniel, D. R. Edwards, P. A. Moore, and D. H. Pote. 1998. Use of grass filter strips to reduce 17ß-estradiol in runoff from fescue-applied poultry litter. J. Soil Water Conserv. 53:74-77.

6. Raman, D. R., A. C. Layton, L. B. Moody, J. P. Easter, G. S. Sayler, R. T. Burns, and M. D. Mullen. 2001. Degradation of estrogens in dairy waste solids: Effects of acidification and temperature. Trans. ASAE. 44:1881-1888.

7. Shore, L. S., M. Gurevitz, and M. Shemesh. 1993. Estrogen as an environmental pollutant. Bull. Environ. Contam. Toxicol. 51:361-366.

8. Shore, L. S., D.L. Correll, and P.K. Chakraborty. 1995. Relationship of fertilization with chicken manure and concentrations of estrogens in small streams. p. 155-162. *In* K. Steele (ed.) Animal waste and the land-water interface. Lewis Publ., Boca Raton, FL.

9. Oberdorster, E. and A. O. Cheek. 2001. Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. Environ. Toxicol. Chem. 20:23-36.

10. Tyler, C. R., S. Jobling, and J. P. Sumpter. 1998. Endocrine disruption in wildlife: A critical review of the evidence. Crit. Rev. Toxicol. 28:319-361.

11. Panter, G. H., R. S. Thompson, and J. P. Sumpter. 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to

environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquat. Toxicol. 42:243-253.

12. Panter, G. H., R. S. Thompson, and J. P. Sumpter. 2000. Intermittent exposure of fish to estradiol. Environ. Sci. Technol. 34:2756-2760.

13. Irwin, L. K., S. Gray, and E. Oberdorster. 2001. Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. Aquat. Toxicol. 55:49-60.

14. Neef, G. 1999. Steroidal estrogens. p. 17-41. *In* M. Oettel and E. Schillinger (eds.) Estrogens and Antiestrogens I. Handb. Exp. Pharmacol. 135. Springer-Verlag Berlin. 135.

15. Tapiero, H., G. N. Ba, and K. D. Tew. 2002. Estrogens and environmental estrogens. Biomed. Pharmacother. 56:36-44.

16. Salole, E. G. 1987. The physicochemical properties of estradiol. J. Pharm. Biomed. Anal. 5:635-648.

17. Kubli-Garfias, C. 1998. Comparative study of the electronic structure of estradiol, epiestradiol and estrone by ab initio theory. Theochem. 452:175-183.

18. Tabak, H. H., R. N. Bloomhuff, and R. L. Bunch. 1981. Steroid hormones as water pollutants 2. Studies on the persistence and stability of natural urinary and synthetic ovulation inhibiting hormones in untreated and treated wastewaters. Dev. Ind. Microbiol. 22:497-519.

19. Hurwitz, A. R. and S. T. Liu. 1977. Determination of aqueous solubility and pka values of estrogens. J. Pharm. Sci. 66:624-627.

20. Batra, S. 1975. Aqueous solubility of steroid-hormones: An explanation for discrepancy in published data. J. Pharm. Pharmacol. 27:777-779.

21. Hahnel, R. 1971. Interactions of estradiol-17ß with amino acids. J. Steroid Biochem. 2:61-65.

22. Lundberg, B., T. Lovgren, and B. Heikius. 1979. Simultaneous solubilization of steroid hormones 2. Androgens and estrogens. J. Pharm. Sci. 68:542-545.

23. Blomquist, C. and L. Sjoblom. 1964. Further studies on the solubilisation of oestrogens. Acta Chem. Scand. 18:2404-2405.

24. Lovgren, T., B. Heikius, B. Lundberg, and L. Sjoblom. 1978. Simultaneous solubilization of steroid hormones 1. Estrogens and C21 steroids. J. Pharm. Sci. 67:1419-1422.

25. Ruchelman, M. W. 1967. Solubility studies of estrone in organic solvents using gas-

liquid chromatography. Anal. Biochem. 19:98-108.

26. Ruchelman, M. W. and P. Haines. 1967. Solubility studies of estradiol in organic solvents using gas-liquid chromatography. J. Gas Chromatogr. 5:290-296.

27. Doisy, E. A. Jr., M. N. Huffman, S. A. Thayer, and E. A. Doisy. 1941. Solubilities of some estrogens. J. Biol. Chem. 138:283-285.

28. Mather, A. 1942. Distributions of estrogens between immiscible solvents. J. Biol. Chem. 144:617-623.

29. Lai, K. M., K. L. Johnson, M. D. Scrimshaw, and J. N. Lester. 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environ. Sci. Technol. 34:3890-3894.

30. Lai, K. M., M. D. Scrimshaw, and J. N. Lester. 2002. Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. Sci. Total Environ. 289:159-168.

31. Holthaus, K. I. E., A. C. Johnson, M. D. Jurgens, R. J. Williams, J. J. L. Smith, and J. E. Carter. 2002. The potential for estradiol and ethinylestradiol to sorb to suspended and bed-sediments in some English rivers. Environ. Toxicol. Chem. 21:2526-2535.

32. Furhacker, M., A. Breithofer, and A. Jungbauer. 1999. 17ß-Estradiol: Behavior during waste water analyses. Chemosphere. 39:1903-1909.

33. Lewis, K. M. and R. D. Archer. 1979. Pka values of estrone, 17ß-estradiol and 2-methoxyestrone. Steroids. 34:485-499.

34. Ingerslev, F. and B. Halling-Sorenson. 2003. Evaluation of analytical chemical methods for detection of estrogens in the environment. Working Report No. 44. Danish Environmental Protection Agency, Danish Ministry of the Environment, Copenhagen, Denmark. 69p.

35. Groh, H., K. Schade, and C. Horholdschubert. 1993. Steroid metabolism with intestinal microorganisms. J. Basic Microbiol. 33:59-72.

36. Lombardi, P., B. Goldin, E. Boutin, and S. L. Gorbach. 1978. Metabolism of androgens and estrogens by human fecal microorganisms. J. Steroid Biochem. Mol. Biol. 9:795-801.

37. Jarvenpaa, P., T. Kosunen, T. Fotsis, and H. Adlercreutz. 1980. Invitro metabolism of estrogens by isolated intestinal microorganisms and by human fecal microflora. J. Steroid Biochem. Mol. Biol. 13:345-349.

38. Williamson, J., D. Vanorden, and J. P. Rosazza. 1985. Microbiological hydroxylation of estradiol: Formation of 2-hydroxyestradiol and 4-hydroxyestradiol

by *Aspergillus alliaceus*. Appl. Environ. Microbiol. 49:563-567.

39. Zondek, B. and J. Sklow. 1942. An enzyme which inactivates estrone. Proc. Soc. Exp. Biol. Med. 49:629-632.

40. Woods, G. F. 1975. Chemical and microbiological transformation of steroids. p. 5-10. *In* E.H.D.Cameron,S.G. Hillier, and K. Griffiths (eds.). Steroid immunoassay. Cardiff: Alpha Omega. Publishing LTD.

41. Bishop, C. M. and M. R. Hall. 1991. Noninvasive monitoring of avian reproduction by simplified fecal steroid analysis. J. Zoo. 224:649-668.

42. Choi, H. S., E. Kiesenhofer, H. Gantner, J. Hois, and E. Bamberg. 1987. Pregnancy diagnosis in sows by estimation of estrogens in blood, urine or feces. Anim. Reprod. Sci. 15:209-216.

43. Mostl, E., H. S. Choi, W. Wurm, M. N. Ismail, and E. Bamberg. 1984. Pregnancy diagnosis in cows and heifers by determination of estradiol-17α in feces. Br. Vet. J. 140:287-291.

44. Szenci, O., R. Palme, M. A. M. Taverne, J. Varga, N. Meersma, and E. Wissink. 1997. Evaluation of false ultrasonographic pregnancy diagnoses in sows by measuring the concentration of unconjugated estrogens in feces. Theriogenology. 48:873-882.

45. Terio, K. A., J. L. Brown, R. Moreland, and L. Munson. 2002. Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biol. 21:215-222.

46. Vos, E. A., R. van Oord, M. A. M. Taverne, and Th. A. M. Kruip. 1999. Pregnancy diagnosis in sows: direct ELISA for estrone in feces and its prospects for an on-farm test, in comparison to ultrasonography. Theriogenology. 51:829-840.

47. Baronti, C., R. Curini, G. D'Ascenzo, A. Di Corcia, A. Gentili, and R. Samperi. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. 34:5059-5066.

48. Jurgens., M. D., R. J. Williams, and A. C. Johnson. 1999. Fate and behaviour of steroid oestrogens in rivers:A scoping study. R&D Technical Report P161. Environment Agency. Bristol. UK.

49. Kushinsky, S. and M. Anderson. 1974. Creepage of estrogens vs loss by sorption on glassware. Clin. Chem. 20:1528-1534.

50. Cohen, S. L., P. Ho, Y. Suzuki, and F. E. Alspector. 1978. Preparation of pregnancy urine for an estrogen profile. Steroids. 32:279-293.

51. Fotsis, T. and H. Adlercreutz. 1987. The multicomponent analysis of estrogens in urine by ion- exchange chromatography and GC-MS .1. Quantitation of estrogens after initial hydrolysis of conjugates. J. Steroid Biochem. 28:203-213.

52. Jarvenpaa, P., T. Fotsis, and H. Adlercreutz. 1979. Ion-exchange purification of estrogens. J. Steroid Biochem. Mol. Biol. 11:1583-1588.

53. Coyotupa, J., K. Kinoshita, R. Y. Ho, C. Chan, W. Paul, M. Foote, and S. Kushinsky. 1970. Variable decomposition by environmental contaminants in air of estrogens on glass plates coated with silica gel for thin-layer chromatography. Anal. Biochem. 34:71-73.

54. Doerr, P. 1971. Thin-layer chromatography and elution of picogram amounts of estradiol. J. Chromatogr. 59:452-456.

55. Kushinsky, S. 1972. Stability of estrogens to oxygen during exposure on silica-gel. J. Chromatogr. 71:161-164.

56. Bain, J. D., L. H. Kasman, A. B. Bercovitz, and B. L. Lasley. 1984. A comparison of three methods of hydrolysis for estrogen conjugates. Steroids. 43:603-619.

57. Graef, V., E. Furuya, and O. Nishikaze. 1977. Hydrolysis of steroid glucuronides with beta-glucuronidase preparations from bovine liver, *Helix pomatia* and *Escherichia coli*. Clin. Chem. 23:532-535.

58. Moore, A. B., G. D. Bottoms, G. L. Coppoc, R. C. Pohland, and O. F. Roesel. 1982. Metabolism of estrogens in the gastrointestinal tract of swine 1. Instilled estradiol. J. Anim. Sci. 55:124-134.

59. Tang, P. W. and D. L. Crone. 1989. A new method for hydrolyzing sulfate and glucuronyl conjugates of steroids. Anal. Biochem. 182:289-294.

60. Carignan, G. and B. A. Lodge. 1980. Comparison of acidic and enzymatic hydrolysis procedures for identification of natural estrogens in pharmaceutical preparations. J. Pharm. Sci. 69:1453-1454.

61. Roos, R. W. and C. A. Laucam. 1985. Liquid chromatographic analysis of conjugated and esterified estrogens in tablets. J. Pharm. Sci. 74:201-204.

62. Kotiyan, P. N. and P. R. Vavia. 2000. Stability indicating HPTLC method for the estimation of estradiol. J. Pharmac. Biomed. Anal. 22:667-671.

63. Velle, W. 1958. Studies on oestrogens in cattle: Urinary oestrogen excretion by the newborn calf. Acta Endocrinol. 29:381-394.

64. Colucci, M. S., H. Bork, and E. Topp. 2001. Persistence of estrogenic hormones in agricultural soils: I. 17ß-estradiol and estrone. J. Environ. Qual. 30:2070-2076.

65. Desaulniers, D. M., A. K. Goff, K. J. Betteridge, J. E. Rowell, and P. F. Flood. 1989. Reproductive hormone concentrations in feces during the estrous-cycle and pregnancy in cattle (*Bos taurus*) and muskoxen (*Ovibos moschatus*). Can. J. Zool. 67:1148-1154.

66. Korner, W., U. Bolz, W. Sussmuth, G. Hiller, W. Schuller, V. Hanf, and H. Hagenmaier. 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere. 40:1131-1142.

67. Lopez de Alda, M. J. and D. Barcelo. 2001. Use of solid-phase extraction in various of its modalities for sample preparation in the determination of estrogens and progestagens in sediment and water. J. Chromatogr. A. 938:145-153.

68. Dizer, H., B. Fischer, I. Sepulveda, E. Loffredo, N. Senesi, F. Santana, and P. D. Hansen. 2002. Estrogenic effect of leachates and soil extracts from lysimeters spiked with sewage sludge and reference endocrine disrupters. Environ. Toxicol. 17:105-112.

69. Tabak, H. H. and R. L. Bunch. 1970. Steroid hormones as water pollutants. I. Metabolism of natural and synthetic ovulation-inhibiting hormones by microorganisms of activated sludge and primary settled sewage . Dev. Ind. Microbiol. 11:367-376.

70. Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ. Sci. Technol. 32:1549-1558.

71. Johnson, A. C., A. Belfroid, and A. Di Corcia. 2000. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci. Total Environ. 256:163-173.

72. Belfroid, A. C., A. Van der Horst, A. D. Vethaak, A. J. Schafer, G. B. J. Rijs, J. Wegener, and W. P. Cofino. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Sci. Total Environ. 225:101-108.

73. Huang, C. H. and D. L. Sedlak. 2001. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environ. Toxicol. Chem. 20:133-139.

74. Korner, W., V. Hanf, W. Schuller, C. Kempter, J. Metzger, and H. Hagenmaier. 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Sci. Total Environ. 225:33-48.

75. Kuch, H. M. and K. Ballschmiter. 2000. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-

MS in the picogram per liter range. Environ. Sci. Technol. 35:3201-3206.

76. Murk, A. J., J. Legler, M. M. H. Van Lipzig, J. H. N. Meerman, A. C. Belfroid, A. Spenkelink, B. Van Der Burg, G. B. J. Rijs, and D. Vethaak. 2002. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. Environ. Toxicol. Chem. 21:16-23.

77. Seibert, D. S. and C. F. Poole. 1998. A general model for the optimization of sample processing conditions by solid-phase extraction applied to the isolation of estrogens from urine. J. High Res. Chromatogr. 21:481-490.

78. Snyder, S. A., T. L. Keith, D. A. Verbrugge, E. M. Snyder, T. S. Gross, K. Kannan, and J. P. Giesy. 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. Environ. Sci. Technol. 33:2814-2820.

79. Spengler, P., W. Korner, and J. W. Metzger. 2001. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany 1. Chemical analysis. Environ. Toxicol. Chem. 20:2133-2141.

80. Ternes, T. A., M. Stumpf, J. Mueller, K. Haberer, R. D. Wilken, and M. Servos. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225:81-90.

81. Petrovic, M., E. Eljarrat, M. J. Lopez de Alda, and D. Barcelo. 2002. Recent advances in the mass spectrometric analysis related to endocrine disrupting compounds in aquatic environmental samples. J. Chromatogr. A. 974:23-51.

82. Hennion, M. C. 1999. Solid phase extraction: Method development, sorbents, and coupling with liquid chromatography. J. Chromatogr. A. 856:3-54.

83. Erb, R. E., R. D. Randel, T. N. Mellin, and V. L. Jr. Estergreen. 1968. Urinary estrogen excretion rates during pregnancy in bovine. J. Dairy Sci. 51:416-419.

84. Abraham, G. E. 1974. Radioimmunoassay of steroids in biological-materials. Acta Endocrinol. 75:7-42.

85. Marchand, P., B. le Bizec, C. Gade, F. Monteau, and F. Andre. 2000. Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. J. Chromatogr. A. 867:219-233.

86. Friedgood, H. B., J. B. Garst, and A. J. Haagensmit. 1948. New method for the separation of androgens from estrogens and for the partition of estriol from estrone-estradiol fraction. J. Biol. Chem. 174:523-554.

87. Kober, S. 1931. A colorimetric determination of the sex hormone (menformone). Biochem. Z. 239:209.

88. Brown, J. B. 1955. Chemical method for the determination of oestriol, oestrone and

oestradiol in Human Urine. Biochem. J. 60:185-193.

89. Chan, A. H. H. and R. H. Common. 1972. A note on the four hour excretion of estrone and estradiol-17ß in the urine of the hen. Poultry Sci. 51:1772-1775.

90. Common, R. H., L. Ainsworth, F. Hertelendy, and R. S. Mathur. 1965. The estrone content of hens urine. Can. J. Biochem. 43:539-547.

91. Ittrich, G. 1958. Eine neue method fur die chemische bestimmung der oestrogenischen hormonen in urin. Zeit. Physiol. 312:1-14.

92. Lunaas, T. 1962. Urinary oestrogen levels in sow during oestrous cycle and early pregnancy. J. Reprod. Fertil. 4:13-20.

93. Lunaas, T. 1965. Urinary excretion of oestrone and oestradiol and of zimmermann chromogens in sow during oestrus. Acta Vet. Scand. 6:16-29.

94. Mathur, R. S. and R. H. Common. 1967. Chromatographic identification of estriol and 16,17-epiestriol as constituents of urine of laying hens. Can. J. Biochem. 45:531-539.

95. Mathur, R. S. and R. H. Common. 1969. A note on the daily urinary excretion of estradiol-17ß and estrone by hens. Poultry Sci. 48:100-104.

96. Mathur, R. S., P.A. Anastassiadis, and R. H. Common. 1966. Urinary excretion of estrone and of 16-epi-estriol plus 17-epi-estriol by hens. Poultry Sci. 45:946-952.

97. Zimmermann, W. 1936. Colorimetrische bestimmung der keimdrusenhormone. Z. Phsyiol. Chem. 245:47.

98. Cohen, S. L. 1969. Removal of substances interfering with the rapid assay of estrogen in pregnancy urine. J. Clin. Endocrinol. Metab. 29:47-54.

99. Mellin, T. N., R. E. Erb, and V. L. Estergreen. 1965. Quantitative estimation and identification of estrogens in bovine urine. J. Dairy Sci. 48:895-902.

100. Raeside, J. I. 1963. Urinary oestrogen excretion in pig at oestrus and during oestrous cycle. J. Reprod. Fertil. 6:421-426.

101. Jones, P. H. and R. E. Erb. 1967. Modified procedure for estimating estrogens in urine. J. Dairy Sci. 50:772-774.

102. Tang, F. Y., T. M. Huston, and H. M. Edwards. 1970. Effects of different environmental temperatures on urinary estrogens of maturing fowl. Poultry Sci. 49:66-76.

103. Fine, D. D., G. P. Breidenbach, T. L. Price, and S. R. Hutchins. 2003. Quantitation of estrogens in ground water and swine lagoon samples using solid-phase

extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography-negative ion chemical ionization tandem mass spectrometry. J. Chromatogr. A. 1017:167-185.

104. Lopez de Alda, M. J. and D. Barcelo. 2001. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disruptors in water by fully automated on-line solid-phase extraction liquid chromatography-diode array detection. J. Chromatogr. A. 911:203-210.

105. Lagana, A., G. Fago, A. Marino, and D. Santarelli. 2001. Liquid chromatography tandem mass spectrometry applied to the analysis of natural and synthetic steroids in environmental waters. Anal. Lett. 34:913-926.

106. Lee, H. B. and T. E. Peart. 1998. Determination of 17ß-estradiol and its metabolites in sewage effluent by solid phase extraction and gas chromatography mass spectrometry. J. AOAC Int. 81:1209-1216.

107. Andersen, H., H. Siegrist, B. Halling-Sorensen, and T. A. Ternes. 2003. Fate of estrogens in a municipal sewage treatment plant. Environ. Sci. Technol. 37:4021-4026.

108. Nakamura, S., T. H. Sian, and S. Daishima. 2001. Determination of estrogens in river water by gas chromatography-negative-ion chemical-ionization mass spectrometry. J. Chromatogr. A. 919:275-282.

109. Shimada, K., K. Mitamura, and T. Higashi. 2001. Gas chromatography and high performance liquid chromatography of natural steroids. J. Chromatogr. A. 935:141-172.

110. Ferguson, P. L., C. R. Iden, A. E. Mcelroy, and B. J. Brownawell. 2001. Determination of steroid estrogens in wastewater by immunoaffinity extraction coupled with HPLC-Electrospray-MS. Anal. Chem. 73:3890-3895.

111. Matsumoto, K., Y. Tsukahara, T. Uemura, K. Tsunoda, H. Kume, S. Kawasaki, J. Tadano, and T. Matsuya. 2002. Highly sensitive time-resolved fluorometric determination of estrogens by high-performance liquid chromatography using a beta-deketonate europium chelate. J. Chromatogr. B. 773:135-142.

112. Wood, W. G. 1991. Matrix effects in immunoassays. Scand. J. Clin. Lab. Invest. 51 (Suppl. 205):105-112.

113. Nunes, G. S., I. A. Toscano, and D. Barcelo. 1998. Analysis of pesticides in food and environmental samples by enzyme-linked immunosorbent assays. Trends Anal. Chem. 17:79-87.

114. Maxey, K. M., K. R. Maddipati, and J. Birkmeier. 1992. Interference in enzyme immunoassays. J. Clin. Immunoass. 15:116-120.

115. Taieb, J., C. Benattar, A. S. Birr, and A. Lindenbaum. 2002. Limitations of steroid determination by direct immunoassay. Clin. Chem. 48:583-585.

116. Balaguer, P., F. Francois, F. Comunale, H. Fenet, A. M. Boussioux, M. Pons, J. C. Nicolas, and C. Casellas. 1999. Reporter cell lines to study the estrogenic effects of xenoestrogens. Sci. Total Environ. 233:47-56.

117. Korner, W., P. Spengler, U. Bolz, W. Schuller, V. Hanf, and J. W. Metzger. 2001. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. Environ. Toxicol. Chem. 20:2142-2151.

118. Legler, J., M. Dennekamp, A. D. Vethaak, A. Brouwer, J. H. Koeman, B. van der Burg, and A. J. Murk. 2002. Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. Sci. Total Environ. 293:69-83.

119. Routledge, E. J. and J. P. Sumpter. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem. 15:241-248.

120. Andersen, H. R., A. M. Andersson, S. F. Arnold, H. Autrup, M. Barfoed, N. A. Beresford, P. Bjerregaard, L. B. Christiansen, B. Gissel, R. Hummel, E. B. Jorgensen, B. Korsgaard, R. Le Guevel, H. Leffers, J. Mclachlan, A. Moller, J. B. Nielsen, N. Olea, A. Oles-Karasko, F. Pakdel, K. L. Pedersen, P. Perez, N. E. Skakkebaek, C. Sonnenschein, A. M. Soto, J. P. Sumpter, S. M. Thorpe, and P. Grandjean. 1999. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ. Health Persp. 107:89-108.

121. Soto, A. M., C. Sonnenschein, K. L. Chung, M. F. Fernandez, N. Olea, and F. O. Serrano. 1995. The E-Screen assay as a tool to identify estrogens - an update on estrogenic environmental pollutants. Environ. Health Persp. 103:113-122.

122. Gray, L. E., W. R. Kelce, T. Wiese, R. Tyl, K. Gaido, J. Cook, G. Klinefelter, D. Desaulniers, E. Wilson, T. Zacharewski, C. Waller, P. Foster, J. Laskey, J. Reel, J. Giesy, S. Laws, J. Mclachlan, W. Breslin, R. Cooper, R. Digiulio, R. Johnson, R. Purdy, E. Mihaich, S. Safe, C. Sonnenschein, W. Welshons, R. Miller, S. Mcmaster, and T. Colborn. 1997. Endocrine screening methods workshop report: Detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reprod. Toxicol. 11:719-750.

123. Tanghe, T., G. Devriese, and W. Verstraete. 1999. Nonylphenol and estrogenic activity in aquatic environmental samples. J. Environ. Qual. 28:702-709.

124. Burnison, B. K., A. Hartmann, A. Lister, M. R. Servos, T. Ternes, and G. Van Der Kraak. 2003. A toxicity identification evaluation approach to studying estrogenic substances in hog manure and agricultural runoff. Environ. Toxicol. Chem. 22:2243-2250.

125. Dorfman, R. I. and A. S. Dorfman. 1954. Estrogen assays using the rat uterus. Endocrinol. 55:65-69.

126. Fishman, J. and C. Martucci. 1980. Dissociation of biological activities in metabolites of estradiol. *p. 131-145. In* J.A. McLachlan (ed.) Estrogens in the environment. Elsevier North Holland. New York.

127. Odum, J., P. A. Lefevre, S. Tittensor, D. Paton, E. J. Routledge, N. A. Beresford, J. P. Sumpter, and J. Ashby. 1997. The rodent uterotrophic assay: Critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. Reg. Toxicol. Pharm. 25:176-188.

128. Shelby, M. D., R. R. Newbold, D. B. Tully, K. Chae, and V. L. Davis. 1996. Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. Environ. Health Persp. 104:1296-1300.

129. Calvert, C. C., L. W. Smith, and T. R. Wrenn. 1978. Hormonal activity in poultry excreta processed for livestock feed. Poultry Sci. 57:265-270.

130. Levin, L. 1945. The fecal excretion of estrogens by pregnant cows. J. Biol. Chem. 157:407-411.

131. Bowman, C. J. and N. D. Denslow. 1999. Development and validation of a species- and gene-specific molecular biomarker: Vitellogenin mrna in largemouth bass (*Micropterus salmoides*). Ecotoxicol. 8:399-416.

132. Denslow, N. D., M. C. Chow, K. J. Kroll, and L. Green. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicol. 8:385-398.

133. Folmar, L. C., M. Hemmer, R. Hemmer, C. Bowman, K. Kroll, and N. D. Denslow. 2000. Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. Aquat. Toxicol. 49:77-88.

134. Lutz, I. and W. Kloas. 1999. Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. Sci. Total Environ. 225:49-57.

135. Miles-Richardson, S. R., V. J. Kramer, S. D. Fitzgerald, J. A. Render, B. Yamini, S. J. Barbee, and J. P. Giesy. 1999. Effects of waterborne exposure of 17ß-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales Promelas*). Aquat. Toxicol. 47:129-145.

136. Sumpter, J. P. and S. Jobling. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Persp. 103:173-178.

137. Sumpter, J. P. 1995. Feminized responses in fish to environmental estrogens. Toxicol. Lett. 82-3:737-742.

138. Hemming, J. M., W. T. Waller, M. C. Chow, N. D. Denslow, and B. Venables. 2001. Assessment of the estrogenicity and toxicity of a domestic wastewater effluent flowing through a constructed wetland system using biomarkers in male fathead minnows (Pimephales promelas rafinesque, 1820). Environ. Toxicol. Chem. 20:2268-2275.

139. Metcalfe, C. D., T. L. Metcalfe, Y. Kiparissis, B. G. Koenig, C. Khan, R. J. Hughes, T. R. Croley, R. E. March, and T. Potter. 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese Medaka (*Oryzias Latipes*). Environ. Toxicol. Chem. 20:297-308.

140. Nichols, K. M., S. R. Miles-Richardson, E. M. Snyder, and J. P. Giesy. 1999. Effects of exposure to municipal wastewater in situ on the reproductive physiology of the fathead minnow (*Pimephales Promelas*). Environ.Toxicol. Chem. 18:2001-2012.

141. Rodgers-Gray, T. P., S. Jobling, S. Morris, C. Kelly, S. Kirby, A. Janbakhsh, J. E. Harries, M. J. Waldock, J. P. Sumpter, and C. R. Tyler. 2000. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environ. Sci. Technol. 34:1521-1528.

142. Knight, W. M. 1980. Estrogens administered to food-producing animals: Environmental considerations. p. 391-401. *In* J.A. McLachlan (ed.) Estrogens in the environment. Dev. Toxicol. Environ. Sci. 5. Elsevier/North Holland:New York.

143. Erb, R. E., B. P. Chew, and H. F. Keller. 1977. Relative concentrations of estrogen and progesterone in milk and blood, and excretion of estrogen in urine. J. Anim. Sci. 45:617-626.

144. Hoffmann, B., T. G. Depinho, and G. Schuler. 1997. Determination of free and conjugated oestrogens in peripheral blood plasma, feces and urine of cattle throughout pregnancy. Exp. Clin. Endocrinol. Diabetes. 105:296-303.

145. Ivie, G. W., R. J. Christopher, C. E. Munger, and C. E. Coppock. 1986. Fate and residues of [4-C-14] estradiol-17ß after intramuscular injection into holstein steer calves. J. Anim. Sci. 62:681-690.

146. Mellin, T. N. and R. E. Erb. 1966. Estrogen metabolism and excretion during bovine estrous cycle. Steroids. 7:589-606.

147. Monk, E. L., R. E. Erb, and T. A. Mollett. 1975. Relationships between immunoreactive estrone and estradiol in milk, blood, and urine of dairy cows. J. Dairy Sci. 58:34-40.

148. Ainsworth, L., R. H. Common, and A. L. Carter. 1962. Chromatographic study of some conversion products of estrone-16-C14 in urine and feces of laying hen. Can. J. Biochem. Physiol. 40:123-135.

149. Common, R. H., R. S. Mathur, S. Mulay, and G. O. Henneberry. 1969. Distribution patterns of in vivo conversion products of injected estradiol-17ß-4-14C and estrone-4-14C in urines of nonlaying and laying hen. Can. J. Biochem. 47:539-545.

150. Palme, R., P. Fischer, H. Schildorfer, and M. N. Ismail. 1996. Excretion of infused C14 steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43:43-63.

151. Edgerton, L. A. and R. E. Erb. 1971. Metabolites of progesterone and estrogen in domestic sow urine .1. Effect of pregnancy. J. Anim. Sci. 32:515-524.

152. Schwarzenberger, F., E. Mostl, R. Palme, and E. Bamberg. 1996. Faecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. Anim. Reprod. Sci. 42:515-526.

153. Szenci, O., M. A. M. Taverne, R. Palme, B. Bertoti, and I. Merics. 1993. Evaluation of ultrasonography and the determination of unconjugated estrogen in feces for the diagnosis of pregnancy in pigs. Vet. Rec. 132:510-512.

154. Raeside, J. I. 1965. Urinary excretion of dehydroepiandrosterone and oestrogens by boars. Acta Endocrinol. 50:611-620.

155. Velle, W. 1958. Further investigations on urinary oestrogen excretion by the boar. Acta Endocrinol. 29:395-400.

156. Erb, R. E., W. R. Gomes, R. D. Randel, V. L. Jr. Estergreen, and O. L. Frost. 1968. Effect of ovariectomy on concentration of progesterone in blood plasma and urinary estrogen excretion rate in pregnant bovine. J. Dairy Sci. 51:420-427.

157. ASAE. 1999. Manure production and characteristics. ASAE Standards, 46th Edition; American Society of Agricultural Engineers: St. Joseph, MI. ASAE D384.1 DEC99.

158. Lange, I. G., A. Daxenberger, B. Schiffer, H. Witters, D. Ibarreta, and H. H. D. Meyer. 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environent. Anal. Chim. Acta. 473:27-37.

159. Vos, E. A. 1996. Direct ELISA for estrone measurement in the feces of sows: Prospects for rapid, sow-side pregnancy diagnosis. Theriogenology. 46:211-231.

160. Raeside, J. I. 1963. Urinary oestrogen excretion in pig during pregnancy and parturition. J. Reprod. Fertil. 6:427-431.

161. Williams, E. L. 2002. Survey of estrogen concentrations in dairy and swine waste holding and treatment structures in and around Tennessee. M.S. thesis. University of Tennessee, Knoxville.

162. Ternes, T. A., P. Kreckel, and J. Mueller. 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants II. Aerobic batch experiments with activated sludge. Sci. Total Environ. 225:91-99.

163. D'Ascenzo, G., A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari, and R. Samperi. 2003. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Sci. Total Environ. 302:199-209.

164. Gentili, A., D. Perret, S. Marchese, R. Mastropasqua, R. Curini, and A. Di Corcia. 2002. Analysis of free estrogens and their conjugates in sewage and river waters by solid-phase extraction then liquid chromatography-electrospray-tandem mass spectrometry. Chromatographia. 56:25-32.

165. Shore, L. S., E. Harelmarkowitz, M. Gurevich, and M. Shemesh. 1993. Factors affecting the concentration of testosterone in poultry litter. J. Environ. Sci. Health Part A. 28:1737-1749.

166. Turfitt, G. E. 1947. Microbiological agencies in the degradation of steroids 2. Steroid utilization by the microflora of soils. J. Bacteriol. 54:557-562.

167. Stumm-Zollinger, E. and G. M. Fair. 1965. Biodegradation of steroid hormones. J. Water Poll. Control Fed. 37:1506-1510.

168. Jurgens, M. D., K. I. E. Holthaus, A. C. Johnson, J. J. L. Smith, M. Hetheridge, and R. J. Williams. 2002. The potential for estradiol and ethinylestradiol degradation in english rivers. Environ. Toxicol. Chem. 21:480-488.

169. Lai, K. M., M. D. Scrimshaw, and J. N. Lester. 2002. Biotransformation and bioconcentration of steroid estrogens by *Chlorella vulgaris*. Appl. Environ. Microbiol. 68:859-864.

170. Schlenker, G., W. Muller, and P. Glatzel. 1998. Analysis for the stability of sexual steroids in faeces of cows over 12 weeks. Berl. Muench. Tieraerztl. Wnchenschr. 111:248-252.

171. Schlenker, G., W. Muller, C. Birkelbach, and P. Glatzel. 1999. Experimental investigations into influence of *Escherichia coli* and *Clostridium perfringens* on the steroid estrone. Berl. Muench. Tieraerztl. Wnchenschr. 112:14-17.

172. Schlenker, G., C. Birkelbach, and P. S. Glatzel. 1999. Analysis of influence of temperature on the stability of sexual steroids in faeces of cows. Berl. Muench. Tieraerztl. Wnchenschr. 112:459-464.

173. Payne, D. W. and P. Talalay. 1985. Isolation of novel microbial 3-alpha-hydroxysteroid, 3-beta- hydroxysteroid, and 17-beta-hydroxysteroid dehydrogenases - purification, characterization, and analytical applications of a 17-beta-hydroxysteroid dehydrogenase from an *alcaligenes Sp.* J. Biol. Chem. 260:3648-3655.

174. Rizner, T. L., J. Adamski, and M. Zakelj-Mavric. 2001. Expression of 17 beta-hydroxysteroid dehydrogenases in mesophilic and extremophilic yeast. Steroids. 66:49-54.

175. Rizner, T. L., M. Zakelj-Mavric, A. Plemenitas, and M. Zorko. 1996. Purification and characterization of 17 beta-hydroxysteroid dehydrogenase from the filamentous fungus *Cochliobolus lunatus*. J. Steroid Biochem. Mol. Biol. 59:205-214.

176. Rizner, T. L. and M. Zakelj-Mavric. 2000. Characterization of fungal 17 beta-hydroxysteroid dehydrogenases. Comp. Biochem. Physiol. B. 127:53-63.

177. Coombe, R. G., Y. Y. Tsong, P. B. Hamilton, and C. J. Sih. 1966. Mechanisms of steroid oxidation by microorganisms .X. Oxidative cleavage of estrone. J. Biol. Chem. 241:1587-1595.

178. Graubard, M. and G. Pincus. 1941. The oxidation of estrogens by phenolases. Proc. Nat. Acad. Sci., USA. 27:149-152.

179. Claus, H. and Z. Filip. 1988. Behavior of phenoloxidases in the presence of clays and other soil-related adsorbents. Appl. Microbiol. Biotechnol. 28:506-511.

180. Bollag, J. M., C. M. Chen, J. M. Sarkar, and M. J. Loll. 1987. Extraction and purification of a peroxidase from soil. Soil Biol. Biochem. 19:61-67.

181. Jellinck, P. H., M. Perry, J. Lovsted, and A. M. Newcombe. 1985. Metabolism of estradiol by true and pseudoperoxidases. J. Steroid Biochem. Mol. Biol. 22:699-704.

182. Jellinck, P. H. and S. Cleveland. 1978. Lactoperoxidase-catalyzed oxidation of [4-C-14]estradiol. Can. J. Biochem. 56:203-206.

183. Klebanoff, S. J. and S. J. Segal. 1960. Inactivation of estradiol by peroxidase. J. Biol. Chem. 235:52-55.

184. Matkovics, B. 1973. C-2 and C-4 enzymic and chemical hydroxylation of phenolic steroids. Steroids Lipids Res. 4:153-161.

185. Matkovics, B., S. E. Rajki, and D. G. Szonyi. 1972. Steroids. XX. Non-specific enzymic hydroxylation of aromatic steroids. 19. In vitro hydroxylation and transformations. Steroids Lipids Res. 3:118-124.

186. Norymberski, J. K. 1977. Polymerization of estradiol by potassium ferricyanide and by horseradish peroxidase. FEBS Lett. 76:231-234.

187. Westerfield, W. W. 1940. The inactivation of estrone. Biochem. J. 34:51-58.

188. Zondek, B. and M. Finkelstein. 1945. The relation between tyrosinase and estrinase in potato extracts. Endocrinol. 36:291-296.

189. Graubard, M. and G. Pincus. 1942. Steroid metabolism: Estrogens and phenolases. Endocrinol. 30:265-269.

190. Berry, D. F. and S. A. Boyd. 1984. Oxidative coupling of phenols and anilines by peroxidase: Structure-activity-relationships. Soil Sci. Soc. Am. J. 48:565-569.

191. Martin, J. P. and K. Haider. 1980. A comparison of the use of phenolase and peroxidase for the synthesis of model humic acid-type polymers. Soil Sci. Soc. Am. J. 44:983-988.

192. Perucci, P., C. Casucci, and S. Dumontet. 2000. An improved method to evaluate the O-diphenol oxidase activity of soil. Soil Biol. Biochem. 32:1927-1933.

193. Suzuki, K., H. Hirai, H. Murata, and T. Nishida. 2003. Removal of estrogenic activities of 17beta estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi. Water Research. 37:1972-1975.

194. Lehmann, R. G., H. H. Cheng, and J. B. Harsh. 1987. Oxidation of phenolic acids by soil iron and manganese oxides. Soil Sci. Soc. Am. J. 51:352-356.

195. Shindo, H. and P. M. Huang. 1984. Catalytic effects of manganese(IV), iron(III), aluminum, and silicon-oxides on the formation of phenolic polymers. Soil Sci. Soc. Am. J. 48:927-934.

196. Stone, A. T. and J. J. Morgan. 1984. Reduction and dissolution of manganese(III) and manganese(IV) oxides by organics 2. Survey of the reactivity of organics. Environ. Sci. Technol. 18:617-624.

197. Casey, F. X. M., G. L. Larsen, H. Hakk, and J. Simunek. 2003. Fate and transport of 17ß-estradiol in soil-water Systems. Environ. Sci. Technol. 37:2400-2409.

198. Lee, L. S., T. J. Strock, A. K. Sarmah, and P. S. C. Rao. 2003. Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soils and sediment. Environ. Sci. Technol. 37:4098-4105.

199. Colucci, M. S. and E. Topp. 2001. Dissipation of part-per-trillion concentrations of estrogenic hormones from agricultural soils. Can. J. Soil Sci. 82:335-340.

200. Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36:1202-1211.

201. Peterson, E. W., R. K. Davis, and H. A. Orndorff. 2000. 17ß-Estradiol as an indicator of animal waste contamination in mantled karst aquifers. J. Environ. Qual. 29:826-834.

202. USEPA. 2001. Environmental assessment of proposed revisions to the national

pollutant discharge elimination system regulation and the effluent guidelines for concentrated animal feeding operations. EPA-821-B-01-001. United States Environmental Protection Agency, Washington, DC.

203. Wilkie, A. C., H. F. Castro, K.R. Cubinski, J. M. Owens, and S. C. Yan. 2004. Fixed-film anaerobic digestion of flushed dairy manure after primary treatment: Wastewater production and characterization. Biosyst. Eng. (in press).

204. APHA. 1998. Standard methods for the examination of water and wastewater. 20th Edition. American Public Health Association, Washington, D.C.

205. Meyer, H. H. D., H. Sauerwein, and B. M. Mutayoba. 1990. Immunoaffinity chromatography and a biotin-streptavidin amplified enzyme-immunoassay for sensitive and specific estimation of estradiol-17ß. J. Steroid Biochem. Mol. Biol. 35:263-269.

206. Mares, A., J. DeBoever, G. Stans, E. Bosmans, and F. Kohen. 1995. Synthesis of a novel biotin-estradiol conjugate and its use for the development of a direct, broad range enzyme immunoassay for plasma estradiol. J. Immunol. Methods. 183:211-219.

207. DeBoever, J., A. Mares, G. Stans, E. Bosmans, and F. Kohen. 1995. Comparison of chemiluminescent and chromogenic substrates of alkaline-phosphatase in a direct immunoassay for plasma estradiol. Anal. Chim. Acta. 303:143-148.

208. Rodbard, D. and J. E. Lewald. 1974. Statistical analysis of radioimmunoassays and immunoradiometric labeled antibody assays: A generalized, weighted, iterative least squares method for logistic curve fitting. p. 165-192. *In* Proceedings, radioimmunoassay and related procedures in medicine. 1. International Atomic Energy Agency, Vienna, Austria.

209. Vadlamudi, K., W. D. Stewart, K. J. Fugate, and T. M. Tsakeris. 1991. Performance-Characteristics for an Immunoassay. Scand. J. Clin. Lab. Invest. 51 (Suppl. 205):134-138.

210. SAS Institute. 2000. The SAS System for Windows. release 8.01. SAS Institute, Cary, NC.

211. Pesce, A. J. and J. G. Michael. 1992. Artifacts and limitations of enzyme-immunoassay. J. Immunol. Methods. 150:111-119.

212. Lagana, A., A. Bacaloni, G. Fago, and A. Marino. 2000. Trace analysis of estrogenic chemicals in sewage effluent using liquid chromatography combined with tandem mass spectrometry. Rapid Commun. Mass Spectr. 14:401-407.

213. Andreolini, F., C. Borra, F. Caccamo, A. Dicorcia, and R. Samperi. 1987. Estrogen conjugates in late-pregnancy fluids: Extraction and group separation by a graphitized carbon-black cartridge and quantification by high-performance liquid-

chromatography. Anal. Chem. 59:1720-1725.

214. Dyer, R. P. 2001. Losses of 17ß-estradiol from fields receiving dairy wastewater. M.S. thesis. University of Tennessee, Knoxville.

BIOGRAPHICAL SKETCH

Travis A. Hanselman was born in Oskaloosa, IA, on Christmas day, 1974. He lived in New Sharon, IA, until graduation from North Mahaska High School in May 1993. He completed undergraduate studies at Iowa State University in December 1997 with a B.S. degree in agronomy. In June 1998, he began a Master of Science program in the Soil and Water Science Department of the University of Florida under the direction of D.A. Graetz. He completed the M.S. program in December 2000 and was awarded an Alumni Fellowship by the University of Florida for PhD studies. He completed the Ph.D. program under the direction of D.A. Graetz and A.C. Wilkie in May 2004.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald A. Graetz, Chair
Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ann C. Wilkie, Cochair
Research Associate Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas A. Obreza
Professor of Soil and Water Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nancy D. Denslow
Scientist of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 2004

Dean, College of Agricultural and Life Sciences

Dean, Graduate School